THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XVI DECEMBER, 1935 NO. 6

THE EFFECT OF EXTRACTS OF PANCREAS ON DIFFERENT VIRUSES.

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Received for publication September 18th, 1935.

PANCREATIC preparations have often been found to destroy the pathogenicity of different viruses, and in many cases it has been assumed that this destruction is caused by trypsin. In some cases, however, active trypsin preparations failed to destroy virus pathogenicity, and conflicting reports on the resistance of a virus to treatment with trypsin have appeared in the literature. Since crude pancreatic extracts contain a large number of enzymes besides trypsin, it is impossible to determine by their use alone whether a given virus is trypsin-sensitive or not. The literature to 1933 has been summarized in a previous paper (Pirie, 1933), on the inactivation of the Rous and Fujinami tumour viruses by pancreatic extracts. I concluded in this paper that inactivation of these viruses was not caused either by proteases or by the lipase of the pancreas. More recently Gordon (personal communication) has shown that pancreas extracts destroy the pathogenicity of the Shope rabbit fibroma virus, and he has concluded in this case also that trypsin is not responsible. The present paper records further results which show that constituents of pancreas other than enzymes can destroy virus pathogenicity, and it is hoped that this may help to clear up the confusion existing on the question of the destruction of viruses by proteolytic enzymes, as well as open up a new method for the study of viruses themselves.

Viruses Used in the Experiments.

The hen tumour viruses are tedious to work with, since the result of an experiment may not be clear for a month after the injection of the hen. It was therefore decided that before a detailed fractionation of the pancreas could be made another test virus must be found. Vaccinia virus grown in chick embryo tissue culture was killed—as judged by subsequent subcutaneous inoculation in rabbits—in the same way as the Rous and Fujinami viruses. This had been previously reported by Hirano (1931), and he had concluded that the inactivation was due to lipase. A test on vaccinia takes only four days before a certain result is obtained. I also found that the organism of bovine pleuropneumonia could be used in testing pancreatic extracts for virucidal power. Pleuropneumonia can be centrifuged out of a serum broth

culture easily at 10,000 r.p.m. for half an hour, and if the sediment is rubbed up with saline a suspension of pleuropneumonia is obtained which has an active lactic dehydrogenase. The activity of the enzyme can be tested in the usual way in a Thunberg tube using methylene-blue as the reducible dye. The activity of the enzyme has been shown to be proportional to the titre of the organism (Holmes and Pirie, 1932). It was found that the lactic dehydrogenase of pleuropneumonia suspensions was inhibited by extracts of pancreas, and that the power to inhibit this enzyme ran strictly parallel to the power to kill vaccinia and the tumour viruses. This test is rapid and simple and was therefore used in fractionating the pancreas extracts. Results were always confirmed with vaccinia or the tumour viruses.

Preliminary experiments showed that the virucidal substance in pancreas has the properties of a fairly unstable fatty acid. It is soluble in acetone, precipitated in acid solution, and adsorbed on to protein precipitates at a low pH. It will withstand boiling if any considerable amount of protein is present, but it is destroyed if boiled in a protein-free solution. After acid precipitation it can be redissolved at an alkaline pH, and this process can be repeated many times without any considerable loss. Fatty acids and phosphatides were therefore prepared from dried pig pancreas by the standard methods and tested for virucidal power.

Preparation of Pancreatic Lipins.

30 g. of dried pig pancreas were extracted by shaking with three lots of petrol ether of 200 ml. each. The insoluble protein residue was filtered off and kept in a desiccator. The petrol ether extract was distilled *in vacuo* in a stream of nitrogen. The residue was taken up in ether, and the phosphatides were precipitated by the addition of several volumes of acetone. The precipitated phosphatides were spun off and taken up in ether. Cephalin was precipitated by the addition of alcohol and the precipitate was spun off and dried. Lecithin was prepared from the cephalin residue by cadmium precipitation according to Levene and Rolf (1927). The residue from the acetone precipitation of the phosphatides, consisting of a solution of fat, fatty acids and cholesterol, was evaporated to a small volume *in vacuo*; 200 ml. of *M*/10 NaOH were then added, and the alkaline suspension was extracted with about 400 ml. of ether. The alkaline solution of soaps was separated from the ethereal solution in a separating funnel and the acids were precipitated from it by acidifying and centrifuging. The acids were then dried *in vacuo*. The fat and cholesterol residue was evaporated *in vacuo* to a small volume and finally dried *in vacuo*. The yields were as follows: Cephalin, 0·2 g. or 0·7 p.c.; lecithin, 0·18 g. or 0·6 p.c.; fatty acids, 0·80 g. or 2·6 p.c.; fat residue, 2·0 g. or 6·6 p.c.

Experimental Results.

From a quantitative study of the virucidal activity of whole dried pancreas and of the separated and recombined fractions, it seems probable that the lecithin and fatty acids account for the whole virucidal power. The concentration of fatty acids is about five times that of lecithin in dried pig pancreas, therefore the acids probably are mainly responsible for this action. Pancreas cephalin has no effect. The separate fatty acid and lecithin fractions have a greater virucidal power—in a concentration equal to their concentration in a 2 p.c. extract of whole dried pancreas—than a 2 p.c. extract of pancreas itself. Mixture of the fatty acids or lecithin with the petrol ether residue from their extraction considerably diminished virucidal activity. A high concentration of any protein had a similar action (Table I).

TABLE I.—(a) *Effect of Pancreas on Lactic Dehydrogenase of Pleuropneumonia.*

Each vacuum tube contained 1·0 ml. phosphate buffer pH 7·4, 1·0 ml. *M*/10 sodium lactate, 1·0 ml. suspension of pleuropneumonia, 1·0 ml. of the neutralized test substance and 0·2 ml. 1/5000 methylene-blue. The reduction times are given in minutes.

Control.	Pancreas 1·0%.	Petrol ether residue 1·0%.	Pancreas fatty acids.		Pancreas lecithin.	
8	63	..	(1/20,000)	76	(1/20,000)	70
10	47	11	(1/4000)	∞	..	
10	47	11	(1/20,000)	200	,,	15
11	..	11	,,	200	,,	92
9	19	..	,,	46	,,	36
16	..	..	(1/40,000)	130	..	
11	..	..	(1/6000)	107	(1/6000)	60

(b) *Effect of Pancreas on Vaccinia Virus.*

The vaccinia suspension used was a 1/10 saline dilution of a ground and lightly spun chick embryo tissue culture of vaccinia. Equal volumes of virus and test substance plus 0·2 ml. *M*/1 phosphate buffer pH 7·4 were mixed and incubated at 37° C. for 1 1/2 hours. Dilutions were then made in saline and 0·2 ml. injections given in the shaved skin of a white rabbit. The reactions were read on the fourth day.

	Dilutions.			
	1/200.	1/2000.	1/20,000.	1/200,000.
Control	××××	××××	×××	×××
Pancreas 1·0 p.c.	neg.	neg.	neg.	neg.
Pancreas fatty acids 1/4000	××	(×)	neg.	neg.
Pancreas lecithin 1/20,000	××××	××××	×××	×××
Control	××××	×××	××	××
Pancreas fatty acids 1/2000	neg.	neg.	neg.	neg.
Pancreas lecithin 1/2000	×××	××	neg.	neg.

(c) *Effect of Pancreas on the Fujinami Tumour Virus.*

Equal volumes of a paper pulp and sand filtrate of a 5·0 p.c. saline extract of tumour tissue and the test substance were mixed plus 1·0 ml. of *M*/10 phosphate buffer pH 7·4 and incubated at 37° C. for 2 hours. 1·0 ml. injections were then made into the breasts and legs of Plymouth rock fowls.

Control.	1·0 p.c. pancreas.	Pancreas acids.		Pancreas lecithin.	
××××	neg.	(1/5000)	×	..	
××××	×	,,	×	..	
××××	(×)	,,	×	..	
××××	neg.	,,	neg.	..	
××××	..	(1/2000)	..	(1/2000)	neg.
××××	..	,,	neg.	,,	(×)
××××	..	,,	×	..	
××××	..	,,	(×)	,,	×
××××	..	,,	neg.	,,	neg.
××××	..	,,	×	,,	..

Four crosses denote a tumour of maximum size. One cross in brackets a tumour the size of a pea.

A final concentration of 1/4000 of pancreas acids and of 1/20,000 of pancreas lecithin was approximately equal to their concentration in a 1·0 p.c. saline extract of pancreas.

The results have been confirmed by showing that commercially obtainable fatty acids and lecithin have a similar effect on the viruses. Of the higher fatty acids, only unsaturated ones are virucidal. This may be a solubility effect. Treatment of the mixed fatty acids from pancreas with hydrogen and palladium black reduces their activity (Table II).

TABLE II.—(a) *Effect of Separate Fatty Acids and Lecithin on the Lactic Dehydrogenase of Pleuropneumonia. Conditions as in previous table.*

Control	8	Control	8	Control	12
M/10 caproic	8	M/10 crotonic	10	1/3000 egg lecithin	22
M/10 caprylic	9	M/100 undecylenic	∞		
M/10 pelargonic	120	M/1000 ,,	10	1/3000 egg lysolecithin	41
M/100 ,,	9	1/1000 oleic	85		
M/100 lauric	120	1/10,000 ,,	22		
M/1000 ,,	8	1/1000 linoleic	∞		
M/100 myristic	∞	1/10,000 ,,	80		
M/1000 ,,	12	1/5000 eliadic	100		
M/100 palmitic	8				
M/100 stearic	8				

(b) *Effect of Separate Fatty Acids on Vaccinia. Conditions as in previous table.*

	Dilutions.			
	1/200.	1/2000.	1/20,000.	1/200,000.
Control	××××	×××	××	××
1/1000 oleic	neg.	neg.	neg.	neg.
1/1000 linoleic	neg.	neg.	neg.	neg.
Control	××××	××××	×××	××
1/3000 egg lecithin	××××	××××	×××	×××
1/3000 egg lysolecithin	××××	××××	×××	××
1/5000 oleic	×××	××	neg.	neg.
1/5000 palmitic	×××	×××	××	neg.

(c) *Effect of Separate Fatty Acids on the Fujinami Tumour Virus. Conditions as in previous table.*

Control.	1/3000 stearic.	1/3000 oleic.
××××	××××	××
××××	..	××
××××	××××	neg.

Control.	1/1000 linoleic.	1/1000 oleic.
××××	neg.	neg.
××××	,,	,,
××××	,,	,,
××××	,,	,,
××××	,,	,,

Crystalline trypsin, chymotrypsin and chymotrypsinogen in 0·1 p.c. or in 1·0 p.c. solution do not have any, or at most a very slight, effect on pleuropneumonia, vaccinia or the fowl tumour viruses. The trypsin used was a specimen prepared by Northrop. The activity was tested by Northrop's formol titration method, using casein as substrate, and was found to be [T.U.] mg. P.N. = 0·031. This is about one-sixth of the activity of freshly prepared crystalline trypsin. The residue from the petrol ether extraction of pancreas also contains active trypsin but has no effect on viruses.

Virucidal fatty acids can be extracted from other organs such as heart and liver, although saline extracts of the whole dried organ have no action. This is probably due to the much smaller concentration of fatty acids in organs other than the pancreas. Mixture of the compounds soluble in petrol ether with the protein residue from extraction removes the inhibitory action of the liver and heart acids completely. Extracts of pancreas do not kill vaccinia and the hen tumour viruses instantly, and it is possible that the period of incubation is necessary for the liberation of fatty acids both from fats by the action of lipase and from proteins, which adsorb them, by the action of trypsin. A 10 p.c., by wet weight, filtered extract of pig pancreas which had been obtained shortly after death and had been kept chilled throughout the extraction had no effect on vaccinia or on the Fujinami virus, even after two hours' incubation. Such an extract, owing to its freshness, must have contained a much smaller concentration of fatty acids than the commercial and dried pancreas that was usually used.

DISCUSSION.

The action of surface-active fatty acids on viruses is not unexpected, and in the case of the Rous tumour virus it has already been reported. Begg (1932) found that this virus was killed by 0·125 p.c. sodium oleate *in vitro*. This action, however, has not previously been discussed as a cause of the inactivation of viruses by pancreatic extracts. It seems probable that the confusion in the literature on the effect of proteolytic enzymes on viruses is to be ascribed to the fact that different commercial preparations contain different amounts of fatty acids and lecithin, and therefore will vary in their action on the different viruses. The cause of inactivation of these viruses by fatty acids and lecithin can only be discussed tentatively. In earlier work with pleuropneumonia and agalactia (Pirie and Holmes, 1933), it was found that the destruction of the dehydrogenases always involved the death of the organisms, as judged by cultural experiments. After treatment with fatty acids the respiration of pleuropneumonia in the presence of broth is completely inhibited, therefore it seems justifiable to assume that here, as in the other cases, destruction of the lactic dehydrogenase involves destruction of the organism. By analogy therefore we may think of vaccinia and the tumour viruses as being killed, not only rendered non-pathogenic, by fatty acids or lecithin. In some preliminary tests with *Esch. coli* it was found that the lactic dehydrogenase, whether contained in the organism or separated from it, is far more resistant to fatty acids. Schulman and Rideal (1935) found that a fatty acid and protein complex separated from pancreas replaced

surface films of protein by a surface film of fatty acid. In this case the acid was being adsorbed at an air-liquid interface, while in the case of the viruses a solid-liquid interface is in question. It is possible that the fatty acids are adsorbed on the surface of the viruses and disrupt it to such an extent that the virus is lysed. Such speculations, however, must remain unproved until there is more precise knowledge of the surface structure of the viruses themselves.

SUMMARY.

Fatty acids and lecithin prepared from dried pig pancreas destroy the pathogenicity of vaccinia and the Rous and Fujinami tumour viruses and inhibit the lactic acid dehydrogenase of pleuropneumonia. It is suggested that the reported inactivation of some viruses by trypsin may be due to the use of pancreas preparations containing fatty acids and lecithin. Northrop's crystalline trypsin had no effect on the viruses mentioned.

I have great pleasure in thanking the Medical Research Council for hospitality in the Farm Laboratories at Mill Hill, where all the work with hen tumours has been carried out. I also wish to thank Dr. J. Shulman for the crystalline trypsin preparations, and Dr. W. E. Gye for his advice and interest at all times.

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SCHICK TESTS IN ICELAND.

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Received for publication October 1st, 1935.

In a previous paper (1932) one of us reported the results of Schick tests performed on school-children in Reykjavik, showing an unusually low percentage of diphtheria-immunes, particularly among the youngest age-groups. The steep rise in immunity at 12 years was supposed to be caused by a wave of diphtheria 10 to 11 years before the tests were made.

During the 3 years and 2 months that have elapsed since these tests were made, the town with a population of about 32,000 has been practically free from diphtheria, the records for the last 4 years being :

	Cases of diphtheria.		Cases of diphtheria.
1931	0	1933	2
1932	1	1934	1

In March and April, 1935, Schick tests were made on 2590 children in two schools in Reykjavik. Of these, 2198 tests were fully performed. Persistent pseudo-reactions were, as before, not counted. The toxin was delivered from the Danish Serum Institute as before, and the technique used in performing the tests was also the same as in 1932. The results are, therefore, comparable.

The figures for 1932 and 1935 are shown below :

Age.	1932.			1935.		
	Number tested.	Number Schick-neg.	Percentage Schick-neg.	Number tested.	Number Schick-neg.	Percentage Schick-neg.
8 years	136	19	13·97	315	25	7·94
9 ,,	144	21	14·58	369	29	7·86
10 ,,	158	25	15·82	382	30	7·85
11 ,,	145	25	17·24	407	42	10·32
12 ,,	129	34	26·36	388	51	13·14
13 ,,	98	33	37·50	337	55	16·32

Although immunity to diphtheria was at a low level in 1932, it is seen to have sunk lower still in 1935. The abrupt rise in immunity at 12 years in 1932 is striking when compared with the gradual rise at this age now.

As the view has been held by some authors that diphtheria immunity may be physiological and need not be acquired through infection, the sudden rise in immunity at age 12 seen in 1932 might have been explained by the

commencing activity of physiological factors at puberty. A slight wave of diphtheria passing over Reykjavik in 1920–21 was, however, held to be responsible for the immunity in the higher age-groups, but no proof could be produced at that time.

If physiological immunity was responsible for this rise in immunity in 1932, we should expect a similar feature of the immunity curve now, for there has been no diphtheria epidemic in the meantime to complicate the issue. But there is no such rise; on the contrary, the figures are what would be expected if immunity were chiefly due to acquired infection. For in the absence of antigenic stimuli we should rather expect a decrease in immunity, not only in the same age-group, but also in the same individuals. This is actually what has occurred:

Group 8 years (1932) with 13·97 p.c. Schick-neg. corresponds with group 11 years (1935) with 10·32 p.c. Schick-neg.;

Group 9 years (1932) with 14·58 p.c. Schick-neg. corresponds with group 12 years (1935) with 13·14 p.c. Schick-neg.;

Group 10 years (1932) with 15·82 p.c. Schick-neg. corresponds with group 13 years (1935) with 16·32 p.c. Schick-neg.;

which indicates a fall in the immunity level among the present 11 and 12 years' age-groups, and an increase by ½ p.c. in the 13 years' age-group. This latter increase lies within the limit of error, so that we may say that immunity has not increased in these age-groups during the past 3 years.

SUMMARY.

Schick tests on 2198 children in 1935 are compared with Schick tests performed in 1931–32 in Reykjavik, the town having meanwhile been free from diphtheria. A steep rise in immunity at 12 years' age, found 3 years ago, is not found now, all age-groups displaying lower percentages of immunes than before. The conclusions are drawn that the steep increase in immunity at age 12 found in 1931–32 was caused by a wave of diphtheria in 1920–21, that the low level of immunity found now must be explained by a want of antigenic stimulation, and that physiological constitution only plays a minor rôle in causing immunity.

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AN IMPROVED DROPPING APPARATUS FOR PREPARING THE ANTIGEN SUSPENSIONS FOR THE SIGMA TEST.

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Received for publication October 3rd, 1935.

In the official description of the Sigma test (Dreyer *et. al.*, 1923) an all-glass floating syphon is shown, and a modified arrangement is given by Fildes (1931) which uses the tip of a Dreyer's pipette as part of the syphon fitted to a constant-level apparatus. In each case certain disadvantages are experienced. The all-glass syphon was difficult to balance correctly while floating, and to adjust so that it delivers drops at the correct rate. In addition it is an elaborate piece of glass-blowing and therefore expensive. With the modified arrangement referred to by Fildes a very large volume of saline solution is required, and the tip of the syphon can be used only for this one purpose. Also, the very decided oscillatory disturbance of the surface level in the bottle every time the air bubbles into the flask causes a variation in the rate of dropping. The method for adjusting this rate by slipping the rubber connecting piece up or down the stem is not a very delicate one, and involves some risk of breaking the syphon.

To surmount these objections, and give extra accuracy and reliability, I have designed an arrangement of apparatus which consists of—

(1) An iron retort stand, about 24 in. high, fitted with a ring and a clamp.

(2) A 500 c.c. flat-bottomed flask, fitted with an india-rubber stopper, and a bevelled outlet tube (about 3/8 in. diameter).

(3) A 4 in. U-tube, about 5/8 in. diameter.

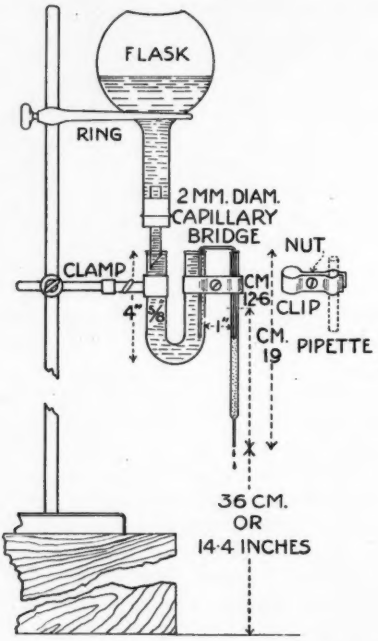
(4) A Dreyer's pipette, held with a clip on the outer limb of the U-tube.

(5) A connecting capillary bridge, 2 mm. diameter, with limbs about 3·5 in. long and 1 in. apart.

The sketch shows the well-known arrangement of apparatus for maintaining a constant level in any vessel, such as the U-tube in this case, and the improvement in the design consists in the use of the complete Dreyer's pipette, which is supported by the U-tube and fed by means of a capillary bridge syphon with saline solution from the U-tube.

The clip for holding the pipette can easily be made of springy hoop iron or brass strip about 1/2 in. wide. It is fitted round the U-tube, and at about 1 in. distance the limbs are bent to embrace the pipette. The grip is obtained by using a small bolt and nut fitted between the two tubes, and tightening it to give a smooth sliding fit, especially on the pipette.

The capillary syphon can be made by using about 8 in. of the central part of the drawn-out glass tube used when making Wright's pipettes, and the diameter (2 mm.) has been adopted by trial as most suitable, especially for the part dipping into the Dreyer's pipette. If much thicker, too little liquid surface is left between the side of the pipette and the syphon to give a good meniscus for judging the level, and if much thinner the syphon does not fill the pipette fast enough to raise the level high enough to deliver the correct amount.



The rate of dropping depends not only on the height of the saline column in the pipette, but also on its viscosity, which varies with the temperature. For the standard rate of dropping the height will therefore vary with the temperature. Experiments were made at different temperatures to determine the height of the saline above the tip of the pipette for this standard rate, and it was found to vary from 11 cm. at 29° C. to 12·55 cm. at about 23° C. These were room temperatures, and the average variation in height was 0·27 cm. for 1° C. For convenience in adjusting the level in the pipette a mark can be made on the stem to suit the usual laboratory temperature, say, at 12·55 cm. above the tip, or if preferred a millimetre scale about 2 cm. long can be used. The clip should be so adjusted as to be about 1/2 in. either above or below the level of the saline in the pipette.

Operating the Apparatus.

The ring and clamp on the stand are arranged so that when the U-tube and inverted flask with saline are in position the fluid fills the U-tube nearly full, say, 1/8 to 1/4 in. below the top. The pipette is then adjusted in the clip which is fixed to the U-tube. The dry capillary bridge is next entered first into the pipette, and then the other limb is dropped into the U-tube. In a 2-mm. tube the saline will rise by surface tension about 1 in., so it at once rises to the bend of the bridge and flows along it into the pipette. Since the inflow at first exceeds the outflow, the saline accumulates in the pipette while its stream of drops is falling out. As the level in the pipette rises the rate of dropping increases, but simultaneously the rate of inflow by the syphon decreases, and thus when the level has risen enough to decrease the inflow till it equals the outflow no further change will be observed in the level, and the pipette then delivers a stream of drops at a constant rate.

When the level comes to the mark or scale on the pipette, the delivery in 4 1/2 minutes is measured in the usual manner with a graduated cylinder and a stop-watch. If it is then found necessary, the pipette can be slipped up or down in its clip till the correct delivery is obtained. The height of fall of the drops can now be regulated by placing the stand on a suitable block or otherwise.

The advantages claimed for this design are :

(*a*) The Dreyer's pipette can be used in preparing the other mixtures for the Sigma test.

(*b*) The capillary bridge adds flexibility, reducing the risk of breakage, and allows easy adjustments in the height of the pipette.

(*c*) The bridge fills itself by capillary action when placed dry into the fluid in the U-tube, and then syphonage fills the pipette and maintains a steady stream of drops.

(*d*) The flow into the pipette may be controlled by removing or replacing the capillary bridge as required.

(*e*) The oscillatory disturbance of level in the U-tube (or other vessel) is scarcely noticeable in the pipette when used with the bridge, so that the level of fluid in it (and therefore the rate of outflow) remains constant during the dropping.

(*f*) The parts take up little space for storage, and are quickly and easily assembled when required for use, or after cleaning.

(*g*) The only special expense is for the Dreyer's pipette, as the other pieces are usually to be found among any laboratory equipment.

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CULTIVATION OF THE VIRUS OF INFLUENZA.

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Received for publication October 9th, 1935.

SINCE the original isolation of a virus from cases of influenza (Smith, Andrewes and Laidlaw, 1933) I have made numerous attempts to cultivate it outside the animal body. In media free from living cells no sign of growth has ever been observed but after many failures successful cultivation was accomplished on the chorio-allantoic membrane of the developing hen's egg. The strain thus obtained was later grown in a medium of minced chicken embryo and Tyrode's solution.

CULTIVATION OF THE VIRUS IN THE DEVELOPING EGG.

In each attempt the original inoculum was a bacteriologically sterile membrane-filtrate prepared from the turbinates or lungs of infected ferrets or from the lungs of infected mice. Fertile eggs of 10–12 days' incubation were inoculated by the technique described by Burnet (1933). Briefly the method consists in dropping virus-containing fluid on the surface of the chorio-allantoic membrane lying at the bottom of an artificially induced air-sac.

Passages from egg to egg were made at intervals of 3 or 4 days. As much as possible of the membrane at the base of the air-sac was removed, minced and emulsified in a measured volume of 50 p.c. broth saline. The supernatant fluid, after either sedimentation or light centrifugation, was used for subculture, infectivity tests and sterility tests. From 6 to 12 eggs were used for each subculture. The tests for infectivity were made on ferrets or mice, the latter species alone being used when titrations of the virus content of cultures were attempted. The sterility tests consisted in allowing a few drops of culture fluid to run down the surface of a blood-agar slope. Anaerobic cultures were not made.

Infectivity of Cultures.

No growth was initiated when virus obtained from ferret turbinates was used.

A culture series, I.C. 11,* originating from a filtrate of lung from a ferret infected with swine influenza virus (Shope, 1934), was made at a time before mice were known to be susceptible to infection. The 2nd, 4th and 7th subcultures were tested on ferrets; both the former produced typical attacks of

* I.C. 11 = Influenza cultures 11th series. /S1, /S2, etc. = number of serial subculture.

ferret influenza, followed by immunity to re-infection, but the latter was inactive. The dilution of original inoculum in the 4th subculture material was approximately 1 in 10^8.

The culture series I.C. 15 was begun by inoculating 6 eggs with a filtrate prepared from the lungs of mice infected with human strain virus. Three or four eggs from each subculture up to and including the 5th (I.C. 15/S5) were separately tested on groups of three mice. One egg was non-infective, but all the others produced typical influenza lung lesions. The dilution of original inoculum at this stage was approximately 1 in 10^{12}.

The membranes of I.C. 15/S6 were used for a titration of virus content. Five membranes were pooled, weighed, emulsified in broth saline and centrifuged. Dilutions of the supernatant fluid were tested on groups of mice. The membranes proved infective in a dilution of 1 in 10,000.

A 5 p.c. emulsion of the membranes of I.C. 15/S8 was centrifuged and the supernatant fluid tested on a ferret. The animal suffered a typical " take ", and was later shown to be immune to reinfection. A filtrate of this emulsion through a 0·7μ gradocol membrane failed to infect either mice or a ferret, but difficulty was experienced in filtration owing to the sticky nature of the emulsion.

The last subculture to be tested was the 10th, which produced typical lung lesions in 4 out of 6 mice. The series was abandoned at this point because of bacterial contamination.

Another culture series, I.C. 16, was titrated on mice at each passage. The infectivity of each culture was evaluated by calculating the degree of dilution of the primary inoculum of virus filtrate on the assumptions that all fluid inoculated into the egg is recoverable when the membranes are excised three or four days later, and that virus suffers no loss of infectivity when kept at 37° C. As a matter of fact a virus filtrate kept in the test-tube at this temperature loses infectivity rapidly, and may be completely inactive after 24 hours. The results obtained with the primary culture, and the first subculture, do not necessarily indicate that growth had occurred in these early stages, but those obtained with the later subcultures provide clear evidence of virus multiplication.

Titre of original filtrate = 1 in 1000.

I.C. 16:	Highest dilution infective	represented	1 in 1000	dilution of original filtrate.
I.C. 16/S1:	,,	,,	,,	1 in 1000 ditto.
I.C. 16/S2:	,,	,,	,,	1 in 20,000 ,,
I.C. 16/S3:	,,	,,	,,	1 in 49,000,000 ,,

Localization of Virus in the Chorio-Allantoic Membrane.

Two eggs of I.C. 16/S4 were used to ascertain whether virus invaded the embryo. A 10 p.c. emulsion of the membranes and a 20 p.c. emulsion of the embryos were made and lightly centrifuged. Each supernatant fluid was tested on 6 mice. All the mice inoculated with membrane emulsion developed typical lung lesions, whereas none of the mice inoculated with embryo emulsion showed any sign of infection.

Absence of Specific Lesions in Membranes.

Many of the egg membranes showed lesions visible to the naked eye, consisting of focal thickenings, either flat opaque patches with a ground-glass appearance or small heaped-up nodules. Such lesions, however, were quite inconstant in infective membranes, and have, moreover, been seen in membranes inoculated with stock broth or saline. Membranes possessing an absolutely normal appearance have proved just as infective as those with lesions.

Many membranes have been examined microscopically. Some have been fixed *in situ* before removal, others have been divided into two equal parts, one part being fixed and the other being proved to be infective for mice. Sections have been stained by hæmatoxylin and eosin and by other methods including Mann's methylblue-eosin stain, which show up brilliantly the inclusion-bodies of many other virus diseases. The microscopical findings fit in well with the naked-eye appearances. Some of the membranes of proven infectivity appeared absolutely normal; others showed changes of non-specific character, such as focal proliferation of ectoderm or endoderm or both, slight increase of keratinization and slight infiltration of the mesoderm with wandering cells, both mononuclear and polymorphonuclear. All these changes have been encountered to an equal extent in membranes which were not infective, and it is clear that finer methods will be needed before the reaction occurring between virus and host cells can be demonstrated.

CULTIVATION OF THE VIRUS IN MINCED CHICK EMBRYO TYRODE SOLUTION MEDIUM.

Minced embryo (8–10-day incubation) was introduced into Carrel flasks by means of a tumour syringe in 0·05 to 0·1 c.c. quantities. The virus inoculum was dropped over the tissue and left for about 10 minutes, then 2 c.c. of Tyrode's solution were added. The tissue mince was distributed by rotation of the flask, which was then capped and incubated at 37° C.

Passages were made at 3- to 4-day intervals. One series was subcultured by transferring 0·05 c.c. of fluid directly from flask to flask, without special precaution for avoiding the transference of small fragments of tissue. A second series was subcultured by grinding the contents of incubated flasks in a mortar with sand and using the supernatant fluid after centrifugation. Either method served equally well; the former has the advantage that it offers far less chance of bacterial contamination.

Infectivity of Cultures.

The primary culture was inoculated with 0·05 c.c. of an emulsion of the membranes of I.C. 16/S3. This emulsion was proved to be infective for mice in a dilution of 1 in 1000. The strain was passed through 6 subcultures when it had to be abandoned owing to the use of a contaminated embryo. The dilution factor for each subculture was approximately 1 in 40 (0·05 c.c. inoculum to 2 c.c. Tyrode's solution + embryo), so that the dilution of original inoculum

in the 6th subculture was over 1 in 10^{11}. Titration of the first few cultures suggested a gradual adaptation of virus to the new medium:

Primary culture was infective in dilution 1 in 10.
1st subculture ,, ,, 1 in 100.
2nd ,, ,, ,, 1 in 1000.

Further comparable titrations were not made as the cultures were then used for other tests, but all were shown to be infective for mice up to and including the last (6th) subculture. The 5th subculture was tested on a ferret and gave a mild attack of the disease.

Association of Virus with the Tissue.

The 4th subcultures were used to determine whether the virus was mainly associated with the tissue or attained a high concentration in the fluid. Three flasks were pooled and centrifuged. The deposit was washed three times with ice-cold Tyrode's solution and the washings were added to the supernatant fluid, which was then made up to a volume of 60 c.c., representing a dilution of 1 in 10 of the fluid portion of the cultures. The washed tissue was transferred to a mortar, ground with sand in 6 c.c. Tyrode's solution, and lightly centrifuged. The centrifuged tissue emulsion and the fluid portion were each titrated on groups of mice, 3 mice being used for each dilution tested. Result:

Tissue emulsion undiluted	+++	++	++
,, ,, 1/10	++	++	+
,, ,, 1/100	++	+	±
,, ,, 1/1000	+	0	0
,, ,, 1/10,000	0	0	0
Fluid of culture 1/10	++	0	0
,, ,, 1/100	0	0	0
,, ,, 1/1000	++	0	0
,, ,, 1/10,000	+	0	0

Signs indicate extent of lung lesions in test mice. +++ = almost complete hepatization; 0 = normal lungs.

The result shows that the virus is closely associated with the tissue. The irregular takes in mice inoculated with the fluid portion were most probably due to small fragments of tissue, which were not thrown down in the centrifuge.

Microscopical Examination of Tissue.

Smears of small pieces of tissue from infective cultures were fixed by wet fixation methods and stained by Giemsa's stain, hæmatoxylin and eosin, and various inclusion-body stains, including Mann's stain. No inclusion bodies were seen, nor was there any appearance which was not also to be found in smears of tissue from control flasks incubated without the addition of virus.

SPECIFICITY OF CULTURE VIRUS.

The evidence that the virus grown was the virus of influenza originally inoculated is as follows:

(1) Culture virus produced typical "influenza" in test ferrets, and the animals were subsequently found to be immune to the stock strain of human influenza virus propagated in ferrets and mice.

(2) Culture virus was neutralized by the anti-influenza horse-serum described by Laidlaw *et al.* (1935).

(3) Control flasks of minced chick embryo and Tyrode's solution, incubated at 37° C. alongside the culture flasks, failed to produce any sign of lung involvement in mice.

(4) The disease produced in mice by culture virus was shown to be transmissible, and had exactly the same features as that produced by the stock strain.

DISCUSSION.

The attempts to cultivate the virus of influenza were made with two main objects. Firstly it was hoped that growth of virus in egg membranes would lead to the development of specific changes, visible both macroscopically and microscopically, such as are produced by many of the viruses which have been propagated by this technique—such changes might afford a useful means of antibody titration of immune sera. Secondly it was considered that culture virus might possess antigenic peculiarities which would help forward the main problem of immunization of man against epidemic influenza. The complete absence of specific lesions and the irregularity of growth in egg membranes makes the egg membrane technique unsuitable for the study of this virus, especially as regards antibody titration, but the successful cultivation in a simple medium of minced chick embryo and Tyrode's solution raises the hope that this method will find some useful application. The antigenic properties of the culture strain are being investigated.

N.B.—Since this paper went to press, Dr. T. Francis, Jr., and Dr. T. P. Magill have reported the successful cultivation of human influenza virus in a medium of minced chick embryo and Tyrode's solution. *Science*, 1935, **82**, 353.

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HÆMOLYTIC STREPTOCOCCAL FIBRINOLYSIS.

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Received for publication October 11th, 1935.

THE phenomenon of hæmolytic streptococcal fibrinolysis was first described by Tillett and Garner (1933, 1934*a* and *b*; Tillett, 1935), who showed that cultures of hæmolytic streptococci possessed the power of dissolving human fibrin clot. These authors stated that the fibrin from individuals suffering from hæmolytic streptococcal infection was resistant to lysis by hæmolytic streptococci, and postulated that this difference from the normal was due to an immunity reaction to such infection. Their work was confirmed by Myers and Keefer (1935).

The present paper records some observations on the phenomenon of fibrinolysis, and compares the findings in normal individuals with those in patients suffering from hæmolytic streptococcal infection, non-streptococcal infections, and non-infective conditions.

TECHNIQUE.

The technique which was adopted is that described by Colebrook and Hare (1934), because it requires only small amounts of plasma and is useful for examining many specimens at the same time.

150 c.mm. of oxalated plasma (2·4 mg. of potassium oxalate to 1 c.c. of blood), diluted 1 in 5 with normal saline, 80 c.mm. of hæmolytic streptococcal culture and 20 c.mm. of 1 p.c. calcium chloride solution were mixed and then incubated at 37° C. The time was taken when a firm clot was established and again when the clot was completely dissolved, the difference in minutes being the "fibrinolysis time". In the case of fibrin which resisted solution a clot might still be present at the end of 24 hours' incubation, at which time the experiment was terminated.

Preliminary work was directed to the standardization of the culture; for two variables were being dealt with, and unless the variation of one of these could be kept within narrow limits no accurate measurement of the other would be possible. With each subculture some slight degradation of the power of the streptococci of producing soluble products occurs. Subculturing was minimized by using dried cultures. A quantity of serum broth culture of the hæmolytic streptococcus in use was prepared, and a drop of the centrifuged deposit was pipetted on to a roll of filter-paper placed at the bottom of a sterile test-tube. The tube and contents were dried in a desiccator containing phosphorus pentoxide; the tube was then attached to a vacuum pump, evacuated to a pressure of a few millimetres of mercury and sealed. Two days before a test the tube was opened, 5 p.c. horse-blood broth added and the whole incubated for 24 hours. The blood broth culture was subcultured into special broth the day before the test, and the 24 hours' growth formed the emulsion used to measure the resistance of the plasmas. In drying the cultures sufficient tubes were prepared for three months' tests. During this period the cultures used were strictly comparable, but had undergone two subculturings more than the cultures used in the previous three months.

At the beginning of the work it appeared possible that resistance to fibrinolysis might depend upon the antigenic structure of the hæmolytic streptococcus producing the fibrinolysin, and that in dealing with large groups of plasmas one culture might give unreliable results (personal communication from Dr. Colebrook). In was therefore decided to use an emulsion prepared by mixing equal quantities of culture of three strains. Two strains, which were kindly supplied by Dr. Colebrook, were selected for their vigorous production of fibrinolysin. They belong to Type 63/T and Type Matthews (Griffiths, 1935), and were isolated from cases of puerperal sepsis. The third strain was also vigorously fibrinolytic, did not react with Griffiths's sera, and had caused an acute tonsillitis in a child.

The broth used was Todd and Hewitt's (1932) medium, and is a glucose bicarbonate, phosphate peptone broth. Peptone broth was prepared by Wright's method (1933), and the glucose and salts, filtered in solution through a Berkefeld filter, were added in correct proportion. Horse-serum was then added to 10 p.c. This medium was selected because ordinary peptone broth, such as was used by Tillett and Garner (1933), does not give a culture with sufficient fibrinolytic activity. During the progress of this work it became apparent that the variability of the culture was chiefly dependent on the broth. In order to obtain consistent results it is desirable to use the Difco peptone recommended by Todd and Hewitt (1932), and to compare a fresh batch of broth with the previous batch.

THE FIBRINOLYTIC REACTION.

Little work has been carried out on the reaction itself. The actual time for fibrinolysis of a specimen of clotted plasma may be a function of some unknown antibody in the plasma, for the measurement in time is not necessarily parallel with the measurement of the known hæmolytic antibody by titration. It therefore was desirable in comparing different plasmas to determine the relation between the fibrinolytic time and the concentration of fibrinolysin employed. This was attempted by using the same plasma but varying the amount of culture used. Also some culture-free fibrinolysin was prepared by the method of adsorption and elution described by Tillett and Garner

TABLE I.—*Relationship Between Concentration of Culture and Fibrinolytic Time.*

Concentration of culture.	Plasma 1.	Plasma 2.	Plasma 3.
100% . 0·080 c.c.	7·0 minutes	8·5 minutes	13·0 minutes
75% . 0·060 ,,	7·0 ,,	9·0 ,,	13·0 ,,
60% . 0·048 ,,	7·5 ,,	17·0 ,,	25·0 ,,
50% . 0·040 ,,	8·5 ,,	23·0 ,,	60·0 ,,
40% . 0·032 ,,	9·5 ,,	80·0 ,,	80·0 ,,
25% . 0·020 ,,	12·5 ,,	88·0 ,,	100·0 ,,
10% . 0·008 ,,	25·0 ,,	140·0 ,,	150·0 ,,

TABLE II.—*Relationship Between Concentration of Fibrinolysin (Culture-free) and Fibrinolytic Time.*

Concentration of fibrinolysin.	Plasma 1.	Plasma 2.	Plasma 3.
100% . 0·080 c.c.	2·0 minutes	4·0 minutes	5·5 minutes
80% . 0·064 ,,	2·0 ,,	4·0 ,,	6·0 ,,
60% . 0·048 ,,	3·0 ,,	18·0 ,,	19·5 ,,
40% . 0·032 ,,	6·0 ,,	66·0 ,,	67·0 ,,
25% . 0·020 ,,	33·0 ,,	..	..

(1934*b*), and dilutions of this were incubated with plasma. The results of these experiments were somewhat unexpected; at first the time increased steadily with decreasing concentration, but with further decrease in concentration a disproportionate increase in fibrinolytic time occurred. The following tables (I, II) and Fig. 1 contain the details of experiments on three normal plasmas. A sharp change in the direction of the curve of fibrinolytic time with concentration occurs at a critical point, which differs for the various plasmas employed.

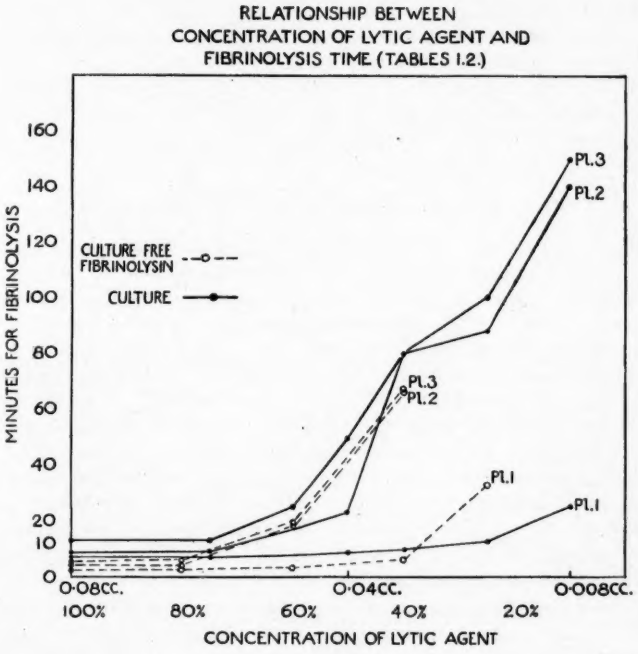

Fig. 1.

From these results it appears that double the time in minutes for fibrinolysis does not imply that the antibody in the plasma is also doubled. The longer the time the greater the concentration of antibody, but to what extent cannot be precisely stated. Further, the effect on the results of using a weak solution of fibrinolysin is shown; the slight differences which exist between normal plasmas are unduly emphasized. It is, however, the gross differences which exist between normal and abnormal plasmas which it is desired to measure. Weak cultures are inadequate to bring out such differences, because when they are used the fibrinolysis times are all of many hours' duration, and the end-points are very difficult to determine. This point is emphasized because the results to be reported differ in some respects from those of other

observers, and this the writer attributes in part to the use of powerfully lytic cultures.

It would also appear from Fig. 1 that the only true measure of any plasma's resistance is the graph of the relation between concentration of fibrinolysin and fibrinolytic time. The construction of such a graph for each plasma is, of course, impracticable when many are being dealt with. The less accurate method already described has to be used. Finally the fact appears that small differences in culture activity might pass unnoticed if a plasma with a very short lysis time were used as a test. This is important when a new batch of broth is being employed.

The phenomenon of great resistance has been studied a little. It was though that such resistance might yield to the employment of fibrinolysin with more bacteria than usual. To this end the centrifuged deposit of 10 c.c. of culture was mixed with 1 c.c. of the supernatant fluid. The effect was to prolong the times for two very resistant plasmas, and not to decrease them.

THE NORMAL RESISTANCE TO FIBRINOLYSIS.

Seventy-two healthy persons have been examined, including 21 boys between the ages of 12 and 15, the remainder being adults. Fig. 2 shows the results, each case appearing as a black square opposite the appropriate time on the chart. It is seen that in the majority of the cases the time fell within 4 to 10 minutes, with a peak at 5 minutes. The average time for the group was 9·1 minutes and the standard deviation was $\pm 14{\cdot}01$ minutes. Since some healthy individuals gave high readings, it was decided to take the mean of the normal group plus three times its standard deviation ($9{\cdot}1 + 3 \times 14{\cdot}01 = 51$ minutes) as the limit for normality. In order to make certain that, owing to the error of determination of lytic times, abnormal plasmas should not wrongly fall within normal limits, the limit might have been increased, for example, by the standard deviation of several determinations on a plasma having an average lytic time of about 50 minutes. An attempt was made to estimate the approximate error in measuring the fibrinolysis time in the case of such partially resistant plasmas. In five plasmas with average fibrinolysis times of 33, 41, 31, 29 and 61 minutes the standard deviation for eight separate determinations on each plasma was $\pm$ 6·5, $\pm$ 6·0, $\pm$ 4·8, $\pm$ 4·4 and $\pm$ 10·2 minutes respectively, while in a normal plasma the standard deviation of eight determinations averaging 9·7 minutes was $\pm$ 0·8 minutes. Thus the accuracy of this method of determining fibrinolysis time diminishes with increase of lytic time. The highest standard deviation found was $\pm$ 10·2 minutes with a plasma having a mean lytic time of 61 minutes. Using this figure, the limit of the normal groups would be $51 + 10 = 61$ minutes. It was felt, however, that the limit was already defined with sufficient stringency, and the figure of 51 minutes was retained.

On the basis of these findings, a division of the plasmas of both normal and abnormal groups was made into—

1. Susceptible—lysis time less than 51 minutes.
2. Partial resistance—greater than 51 minutes, less than 24 hours.
3. " Complete " resistance—absence of or incomplete lysis after 24 hours.

Reference to Fig. 2 will show that this assignment of plasmas would not have been appreciably altered if a limit of 61 minutes had been adopted. It is seen that of 72 normals, 98·6 p.c. fell within the 51 minutes' level and there remained one case outside this level. This case was a young man examined with a view to joining a voluntary blood donor's service. He had been passed as medically fit to give blood. On questioning him further he gave the history of an attack of mastoiditis and "meningitis" at the age of five, with a discharging ear for many years since, and a further abscess in the ear nine years

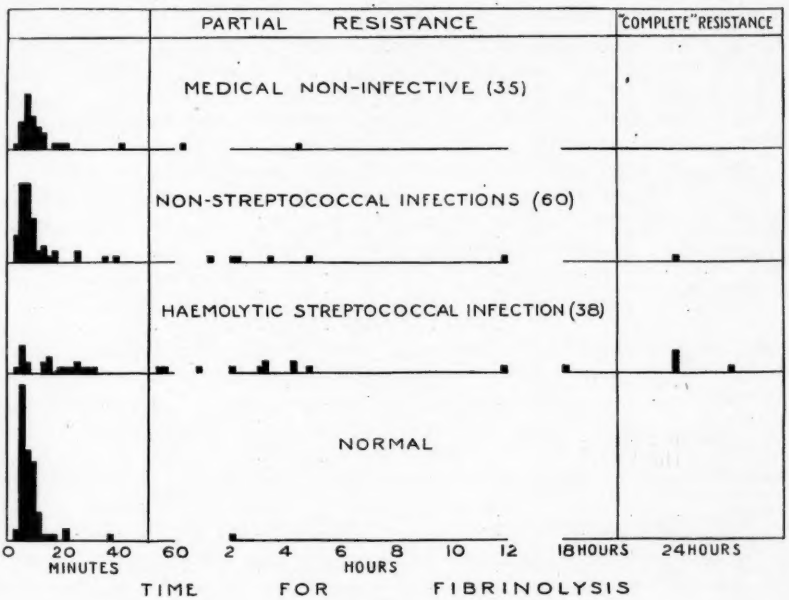

FIG. 2.

before examination. His ear was healed at the time of examination and he had no abnormal physical signs. The history is not incompatible with hæmolytic streptococcal infection and the high figure may be due to past infection, but, in the absence of definite knowledge, the patient must be included in the group of normals.

THE RESISTANCE IN PATIENTS WITH HÆMOLYTIC STREPTOCOCCAL INFECTION.

Thirty-eight patients suffering from various forms of hæmolytic streptococcal infection were examined. Eleven with mastoiditis, from whom hæmolytic streptococci were isolated in pure culture, included one patient with septicæmia and a pyæmic abscess, one with pneumonia, and one with meningitis without organisms in the cerebro-spinal fluid. "Complete" resistance was

found in two of the complicated cases, and partial resistance in three of the others and in the remaining complicated case. The remaining cases were susceptible both shortly after operation and three weeks later. All the patients recovered from the infection.

There were 3 cases with streptococcal cellulitis, in one case secondary to a sore throat with cervical abscess. One was partially and one "completely" resistant. There were 4 cases of erysipelas on whom no cultures were made. Three were susceptible, and the fourth was susceptible on the first occasion, but after the rash had disappeared showed partial resistance.

TABLE III.—*Resistance to Fibrinolysis of Plasmas from Various Groups of Patients.*

Group.	Total number.	Susceptible.		Partial resistance.		"Complete" resistance.	
Normal	72	71	98·6%	1	1·4%	0	
Hæmolytic streptococcal infections:							
Mastoiditis	11	5		4		2	
Cellulitis	3	1		1		1	
Erysipelas	4	3		1		0	
Septicæmia	2	1		1		0	
Tonsillitis and pharyngitis	18	11		5		2	
	38	21	55·3%	12	31·6%	5	13·1%
Non-streptococcal infections:							
Tuberculous	27	24		2		1	
Pneumococcal	4	4		..		..	
Staphylococcal	5	5		..		..	
Osteomyelitis	5	4		1		..	
Diphtheria	5	4		1		..	
Subacute bacterial endocarditis	3	2		1		..	
Syphilitic aortitis	2	1		1		..	
B. coli pyelitis	2	2		..		..	
Typhoid	1	1		..		..	
Meningococcal	2	2		..		..	
Miscellaneous	4	4		..		..	
	60	53	88·3%	6	10·0%	1	1·6%
Medical:							
Gastric ulcer	7	7		..		..	
Ulcerative colitis	1	1		..		..	
Jaundice	2	2		..		..	
Epilepsy	1	1		..		..	
Hypertension	2	2		..		..	
Spontaneous pneumothorax	1	1		..		..	
Bronchiectasis	3	3		..		..	
Psoriasis	1	1		..		..	
Erythema multiforme	1	1		..		..	
Nephritis	3	3		..		..	
Endocrine	1	1		..		..	
Toxic goitre	3	3		..		..	
Miscellaneous	3	3		..		..	
Erythema nodosum	6	4		2		..	
	35	33	94·3%	2	5·7%	0	

Two cases of streptococcal septicæmia included one following puerperal sepsis examined only after the infection had subsided, when the fibrin was susceptible, and one following a finger-prick. In the latter case the fibrin was susceptible during the period of septicæmia, but following the establishment and drainage of an axillary abscess, partial resistance was found.

So far the cases examined were in various stages of the infection, and no detailed attempt had been made to study the time of appearance of the resistance to fibrinolysis in relation to the stage of infection. Tillett (1935) considers that the appearance of resistance coincides with the overcoming by the host of the infective process, and in some of the above patients this appeared to be so, yet others who showed resistance continued after this date to exhibit signs of infection—at any rate of a local character. The remaining patients examined were all suffering from hæmolytic streptococcal infection of the upper respiratory tract (tonsillitis or pharyngitis). In each case hæmolytic streptococci were isolated in predominance from the throat flora. There were 18 patients, of whom 9 were examined in the first week after the onset of the infection (all were susceptible), 13 were examined in the second week (2 with partial resistance), and 11 in the third or fourth weeks (3 with partial resistance, 2 with " complete " resistance). Although the number of cases was small, it appeared probable that the development of resistance following tonsillitis occurred during the second or third weeks after the infection, at which time all clinical signs of infection had disappeared.

On considering all the patients with hæmolytic streptococcal infection without reference to the stage of the disease process at which the blood was examined, 31·6 p.c. showed partial resistance and 13·1 p.c. " complete " resistance. Thus resistance to fibrinolysis is present in the blood of a proportion of individuals suffering from hæmolytic streptococcal infection, but recovery from infection is not always associated with the development of such resistance.

RESISTANCE IN PATIENTS WITH NON-STREPTOCOCCAL INFECTIONS.

To demonstrate that the phenomenon of resistance to fibrinolysis is related to hæmolytic streptococcal infection, it is necessary to show that it develops in this infection, and that it does not develop in infections due to other bacterial agents. To this end 60 cases of infections not due to hæmolytic streptococci have been examined. Table III and Fig. 2 show the findings. Partial resistance was present in 10·0 p.c. and " complete " resistance in 1·6 p.c. In the tuberculous cases, 2 cases of joint tuberculosis and 1 of bone tuberculosis showed resistance. The patient with bone tuberculosis gave a history of acute rheumatism two years previously and had a systolic apical murmur in the heart (partial resistance). One of the patients suffering from joint tuberculosis with " complete " resistance had a discharging sinus with offensive pus and had also recently had tonsillitis, while the third case did not, on history or examination, present any reason for the high figure (partial resistance). One generalized, 4 abdominal and 12 pulmonary tuberculous cases were all susceptible.

Pneumococcal infections included 2 of lobar pneumonia, 1 of mastoiditis due to Type 3 pneumococcus, and 1 of pericarditis due to a pneumococcus (type unknown); all were susceptible.

Five staphylococcal infections—4 cases of osteomyelitis, including one with septicæmia and one of carbuncle—were susceptible, although one case of osteomyelitis had a 40-minute figure. Of 5 other cases of osteomyelitis without cultural evidence, 1 was partially resistant.

Five cases of diphtheria were examined. One fatal case showed partial resistance. This boy died and had diphtheria of the " septic " type, which is so often associated with mixed streptococcal and diphtheritic infection, but no cultures were made at the post-mortem. The other cases were susceptible.

Two cases of syphilitic aortitis were examined. One case was partially resistant, and the patient had had a severe attack of erysipelas a few months

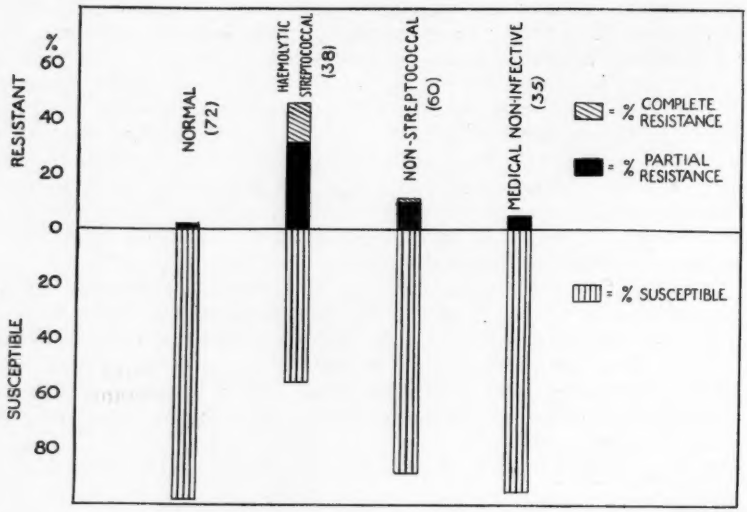

FIG. 3.

previously. Three cases of bacterial endocarditis were examined. In two only was the blood-culture positive (*Streptococcus viridans*). One of the two with positive blood-culture was susceptible on first examination in spite of the fact that he had previously had rheumatic fever. Just before death there was partial resistance, while at death the endocarditis was of infective nature on the mitral and aortic valves, and there was evidence of old rheumatic disease of the mitral valve and myocardium. The second patient with *Streptococcus viridans* infection was susceptible, and had no history or post-mortem evidence of past rheumatism. The third patient with a negative blood-culture was susceptible.

The remaining cases with various infecting agents were susceptible. Their diseases and agents are given in Table III.

Thus in the case of infections not due to the hæmolytic streptococcus, resistance to fibrinolysis does not occur with any frequency. When it is

encountered, evidence of hæmolytic streptococcal infection can sometimes be traced in the past history.

RESISTANCE IN MISCELLANEOUS MEDICAL CASES.

A small number of individuals suffering from ailments of a non-infective nature were examined. Fig. 2 and Table III show that the results were much the same as in the group of normal individuals: 94·3 p.c. of 35 cases were susceptible and 5·7 p.c. were partially resistant. The two patients with resistance were both suffering from erythema nodosum following a sore throat, four further patients with erythema nodosum being susceptible. There were two patients with hæmorrhagic nephritis, neither of whom showed resistance to fibrinolysis. The group includes no case with lesions of a rheumatic nature or with chronic arthritis.

COMPARISON OF FIBRINOLYSIS TIME WITH ANTI-HÆMOLYSIN TITRE.

Todd has shown that in hæmolytic streptococcal infection the blood develops neutralizing substances to streptococcal hæmolysin, and it was therefore considered of interest to attempt a comparison between fibrinolysis time and antihæmolysin titre in a number of instances. This has been carried out by courtesy of Dr. G. J. Griffiths, of the Devonshire Hospital, Buxton, who determined the antihæmolysin titre of blood on which the reaction to fibrinolysin was simultaneously estimated. Table IV shows the findings in 13 patients, and reveals an absence of direct correlation between the antihæmolysin titre and the fibrinolysis time. Thus J. G— and P. C—, with titres of 350 units, had fully susceptible fibrin (7 minutes) and "complete" resistance (no change in 24 hours) respectively. All patients showing resistance to fibrinolysis showed raised antihæmolysin titres, though not necessarily of the same degree.

TABLE IV.—*Comparison of Fibrinolysis Times and Antihæmolysin Titres.*

Patient.	Disease.	Antihæmolysin titre (normal = < 50 units.)	Fibrinolysis time (normal = < 51 minutes).	
			Minutes.	Significance.
M. S—	Streptococcal tonsillitis 12th day	150 units	7	Susceptible.
	Ditto 19th ,,	150 ,,	19	,,
	,, 26th ,,	150 ,,	26	,,
F. S—	,, 5th ,,	85 ,,	12	,,
	,, 12th ,,	150 ,,	1440	"Complete" resistance.
M. P—	Subacute rheumatism	350 ,,	73	Partial resistance.
J. G—	Rheumatoid arthritis	350 ,,	7	Susceptible.
H. McM—	Subacute rheumatism	150 ,,	270	Partial resistance.
J. R—	,, ,,	65 ,,	4	Susceptible.
J. He—	,, ,,	250 ,,	360	Partial resistance.
J. Ho—	,, ,,	250 ,,	360	,, ,,
S. T—	Rheumatoid arthritis	350 ,,	3	Susceptible.
W. H—	Subacute rheumatism	150 ,,	36	,,
F. S—	,, ,,	95 ,,	12	,,
M. E—	Post-scarlatinal carditis	350 ,,	No change in 24 hours	"Complete" resistance.
P. C—	Acute rheumatism	550 ,,	Ditto	,, ,,
	,, ,,	350 ,,	,,	,, ,,

This finding is not inconsistent with the view that resistance to fibrinolysis suggests hæmolytic streptococcal infection, but such infection is not always accompanied by a development of fibrinolytic resistance.

SUMMARY.

1. A technique for measuring the resistance to hæmolytic streptococcal fibrinolysis is described.

2. Resistance to fibrinolysis was present in 1·4 p.c. of normal individuals, 5·7 p.c. of patients suffering from non-infective conditions, 11·6 p.c. of patients with non-streptococcal infections and 44·7 p.c. of patients with proved hæmolytic streptococcal infection. Maximum (" complete ") resistance to fibrinolysis was found in 13·1 p.c. of proved hæmolytic streptococcal infection, 1·6 p.c. of non-streptococcal infection, and not at all in normal and non-infective states.

3. The presence of resistance to fibrinolysis is considered to be related to hæmolytic streptococcal infection, particularly if the resistance is of maximum degree.

4. A comparison of resistance to fibrinolysis and antihæmolysin titre is described.

This work was commenced under the tenure of a Lawrence Scholarship at St. Bartholomew's Hospital ; the expenses of the major portion were defrayed by a grant from the Medical Research Council.

I wish to acknowledge my indebtedness to the Physicians and Surgeons of St. Bartholomew's Hospital for permission to study cases under their care. Dr. Frederick Griffiths, of the Ministry of Health Pathology Laboratory, and Dr. Leonard Colebrook, of Queen Charlotte's Hospital, have given much advice and criticism. Finally I wish to thank Dr. A. A. Miles and Prof. E. H. Kettle, of the British Post-Graduate Medical School, for much helpful advice and criticism.

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TOXICITY AND ANTIGENIC PROPERTIES OF DIFFERENT FRACTIONS OF *B. DYSENTERIÆ* (SHIGA).

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Received for publication October 15th, 1935.

A number of investigators have studied the toxic and immunizing properties of extracts from *B. dysenteriæ* (Shiga). Conradi (1903) recorded that substances extracted from *B. dysenteriæ* with saline at 37° C. during 1 to 2 days were toxic for rabbits. Similar results were obtained by Neisser and Shiga (1903), Kruse (1903), Luedke (1905), Flexner and Sweet (1906) and Klein (1907). Vaillard and Dopter (1906) obtained toxic extracts by suspending bacteria for 20 to 40 days in saline; Luedke (1905) by suspending them in distilled water for 5 days, and Olitsky and Kligler (1920) by extracting in saline for 2 days. On the other hand, Kraus and Doerr (1906) obtained toxic extracts after a short extraction for 1 hour, and according to Kolle, Heller and de Mestral (1908) a short saline extraction for 15 minutes is sufficient to obtain toxic products. Luedke (1905), Besredka (1914) and Fukuhara and Ando (1913) triturated dried bacteria in order to obtain the bacterial substance in a powdered form; saline extracts of these bacterial powders were toxic for rabbits.

Immunization experiments with this substance gave different results according to the different degrees of toxicity and the resulting losses of animals. Conradi (1903) and Neisser and Shiga (1903) reported satisfactory results, while Luedke claimed that it was impossible to produce antibodies with the toxic saline extracts, but that water extracts yielded better results. Selter (1910) reported that extracts prepared at 60° C. were highly toxic and were not useful for immunization. Di Donna (1908) used NaOH for extraction and obtained a toxic product by precipitation with acetic acid. Tsuzuki (1909) used antiformin and with this extract obtained high agglutinin titres, but the losses of animals were heavy.

The purpose of the experiments reported in this paper was to study the toxic and immunizing fractions of the organism, and, if possible, to isolate a substance of low toxic effect and high immunizing power which can be extracted from the bacteria in a quantity sufficient to immunize a large number of animals.

TECHNIQUE.

For the preparation of the various fractions 24-hour cultures of *B. dysenteriæ* (Shiga) were used. The surface growth of 20 culture bottles was washed off with saline, and after centrifugation the bacteria were subjected to the different extractions. The fractions P1, P2 and C were prepared by the method used

by Furth and Landsteiner (1928) for typhoid, paratyphoid and proteus strains; the fractions Q, T and the hapten (Ha) were prepared by the method used by White (1932, 1933) for strains of the Salmonella group. Two further fractions were prepared, as follows: After removing the fractions Q and T the bacteria were boiled for 2 hours in a physiological saline solution, both with and without the addition of 10 p.c. glycerol; then the bacteria were removed by filtration and the dissolved substance in each case precipitated from the filtrate by the addition of 2 volumes of alcohol. The fractions thus obtained were called Gl (glycerol) and Sa (saline). All these fractions were dried in the desiccator, weighed and then suspended in saline so that 1 mg. of the fraction was contained in 1 c.c. of the suspension. From 10 culture flasks the following average quantities of the various extracts were prepared: P1 (first extraction) 5 mg., P1 (second extraction) 8 mg., P2 12 mg., C 18 mg., Q 3 mg., T 20 mg., Gl 2 mg., Sa 3 mg., Ha 24 mg.

As a test culture for infecting the animals after immunization a highly toxic Shiga strain was used. In order to keep the strain virulent it was transplanted daily in broth and transferred to agar slants 24 hours before the test. The surface growth of these 24-hour cultures was washed off with saline solution and used for injection.

RESULTS.

Some Chemical Characteristics of the Various Fractions.

The various fractions were analysed for nitrogen and tested for phosphorus and carbohydrate content. The results obtained with P1 and P2 substances are given in Table I.

TABLE I.

	Percentage of nitrogen.	Hydrolysis.	Reduction of Fehling solution.	P_2O_5.
P1 (crude)	2·3	4% HCl incomplete; 10% HCl complete after 1 hour	Positive	Positive.
P1 (precipitated)	7·3	6% HCl complete after 1 hour	Negative	Slightly positive.
P2 (crude)	1·9	4% HCl complete after 10–15 minutes	Positive	Positive.
P2 (precipitated)	7·8	4% incomplete; 12% HCl complete after 1 hour	Negative	Slightly positive.

It will be noted that the crude extracts contained relatively little N, gave a positive Fehling reaction on hydrolysis and contained P_2O_5, while the purified substances had a higher N content and were free from carbohydrate. The bacteria, after the extraction, did not show any marked loss in nitrogen content, as is demonstrated by the following figures:

(1) Bacteria before extraction	. . . . .	7·2% of nitrogen.
,, after ,,	of P1	8·3% ,,
,, ,, ,,	of P1 and P2 . .	9·6% ,,
(2) Bacteria before extraction	. . . . .	6·9% ,,
,, after ,,	of Q	5·8% ,,
,, ,, ,,	of Q and T . . .	5·6% ,,

The various carbohydrate fractions were different in their chemical composition. The extract made according to the method of Furth and Landsteiner (1928) gave a substance " C " which contained 3·7 p.c. of nitrogen; on the other hand, the biuret-negative hapten prepared according to White (1933) contained only 0·8 p.c. of nitrogen. It is clear, therefore, that the crude extracts really contain mixtures of protein and carbohydrate substances, and that the former can be removed by further purification. The results obtained in the animal experiments should be interpreted on the basis of the chemical composition indicated above.

The Toxicity of the Different Fractions.

Table II gives a summary of the losses of animals resulting from the intravenous injections of the different substances. In the fatal cases the animals showed pronounced intestinal symptoms accompanied by loss of weight of more than 100 g. Paralysis was not observed. In the cases of P1 and P2 the crude extracts as well as the purified substances were tested. The latter were obtained by repeated precipitations with acetic acid, as described by Furth and Landsteiner (1928).

TABLE II.

Substance.	Quantity injected.	Total number of rabbits.	Survived.	Died.
P1 (crude)	0·5 c.c.	4	4	0
	1·0 ,,	11	11	0
	2·0 ,,	4	3	1
P1 (purified)	0·5 ,,	4	4	0
	2·0 ,,	4	2	2
P2 (crude)	0·5 ,,	3	2	1
	1·0 ,,	8	4	4
	2·0 ,,	3	0	3
P2 (purified)	0·5 ,,	3	2	1
	2·0 ,,	3	0	3
Q	1·0 ,,	4	3	1
T	1·0 ,,	7	6	1
Gl	1·0 ,,	3	3	0
Sa	1·0 ,,	2	2	0
C	1·0 ,,	3	3	0
Ha	1·0 ,,	2	2	0

The toxicities of the bacterial residues after the different treatments are shown in Table III.

TABLE III.

Treatment of bacteria.	Number injected.	Number of rabbits.	Survived.	Died.
Extraction of P1	$1{,}000 \times 10^6$	3	1	2
,, P1 and P2	$50{,}000 \times 10^6$	3	2	1
,, Q and T	$10{,}000 \times 10^6$	2	2	0
,, ,,	$50{,}000 \times 10^6$	2	1	1
Untreated bacteria (phenolized)	$1{,}000 \times 10^6$	8	0	8

Similar results were obtained by the method described by Shwartzman (1928) for demonstrating the toxic substances of *B. typhosus*. Four rabbits received intracutaneous injections on different areas of the skin, 0·3 c.c. of P1A (first extraction), P1B (second extraction), P2 and C respectively; 24 hours later each rabbit received an intravenous injection of 1 c.c. of one of these substances. On the following day the animals showed lesions at the points of the intracutaneous injections (Shwartzman phenomenon), as shown in Table IV.

TABLE IV.

Rabbit No.	Intravenous injection of—	Size of the skin lesion in mm. at the point of intracutaneous injection with the fraction :			
		P1A	P1B	P2	C
1	P1A	0	3×5	4×9	0
2	P1B	4×4	2×5	23×15	5×5
3	P2	4×9	3×5	13×16	5×4
4	C	0	5×5	5×4	0

A corresponding experiment was undertaken with the substances Q, T, Gl and Sa, but toxic effects could not be demonstrated. It is apparent that the P2 fraction is richest in the toxic substance of the Shiga bacillus.

Neutralization of the Toxic Substances by Different Antisera.

A group of 10 rabbits received intracutaneous injections of 0·3 c.c. of P2 as "skin preparatory factor". After 24 hours 5 animals received intravenously 1·5 c.c. of P1B and the other 5 animals 1·5 c.c. of P2 neutralized with 0·2 c.c. of various immune sera. The antigen-serum mixtures were incubated at 37° C. for 30 minutes before the intravenous injection. The neutralizing effect of the different antisera on the "reacting factors" is shown in Table V.

TABLE V.

Substance injected intravenously.	Neutralized with rabbit serum.	Size of the skin lesion in mm. at the point of the intracutaneous injection with P2.
P1B	P1A	0
,,	P1B	0
,,	P2	0
,,	C	5 × 5
,,	Normal serum	12 × 20
P2	P1A	9 × 8
,,	P1B	2 × 5
,,	P2	0
,,	C	18 × 8
,,	Normal serum	22 × 25

Protective Effect of Different Antisera for Rabbits.

The rabbits received 1·5 c.c. of a given immune serum intravenously. After 30 minutes they received 1 c.c. of a bacterial suspension containing 1000 × 10^6 organisms per c.c. The results shown in Table VI were obtained.

TABLE VI.

Immune serum injected.	Total number of rabbits.	Survived.	Died.	Loss of weight (g.) 3 days after infection.
P1	2	2	0	0
P2	2	1	1 (after 9 days)	150
C	2	1	1 (after 7 days)	50
Q	2	2	0	75
T	2	2	0	100
Gl	2	2	0	50
Normal serum	2	0	2 (after 3 and 5 days)	..

Protective Effect of Antisera for Mice.

The animals received a subcutaneous injection of 4 c.c. of a given serum. Two days later they received 500 × 10^6 bacteria subcutaneously. The animals which died did so within 2 days after infection. The surviving animals were killed 7 days after infection and cultured according to the technique of Kligler and Olitzki (1930). The results are given in Table VII.

TABLE VII.

Serum injected.	Died.	Blood infected.	Blood sterile, liver and spleen infected.	Local foci.	Sterile.	Total number.	Percentage mortality.
P1	4	2	6	0	3	15	26·7
P2	5	2	2	1	4	14	35·7
P1 and P2 (mixed 1 : 1)	0	2	6	1	3	12	0
Normal serum	10	1	2	1	1	15	66·7

Production of Specific Agglutinins for B. dysenteriæ (*Shiga*) *and of Heterophilic Antibodies with Different Fractions.*

Rabbits were immunized by three intravenous injections of the given fraction at 2-day intervals (0·5, 1·0 and 1·5 c.c.). Seven days after the last injection blood was taken and the serum tested for its agglutinin content for *B. dysenteriæ* (Shiga) and for hæmolysin for sheep cells. The results given in Table VIII were obtained.

TABLE VIII.

Antigen injected.	Quantity of antigen in 1 c.c.	Agglutinin units in 1 c.c.	Hæmolysin units in 1 c.c.
P1 (crude)	1 mg.	500	200
P1 (purified)	1 ,,	200	0
P2 (crude)	1 ,,	100	1000
P2 (purified)	1 ,,	100	500
C	1 ,,	200	100
Q	1 ,,	200	50
T	1 ,,	500	200
Gl	1 ,,	100	200
Sa	1 ,,	100	1000
Ha	1 ,,	50	10
Phenolized bacteria	1,000 × 10^6	1000	2000
Bacteria :			
After removing P1	10,000 × 10^6	1000	500
,, ,, P1 and P2	50,000 × 10^6	500	200
,, ,, Q and T	10,000 × 10^6	200	200

Active Immunization Experiments.

The details of three experiments on active immunization of rabbits with the various fractions are given in the following protocols :

Experiment 1.—Method of immunization : 3 intravenous injections of 1 c.c. of antigen at 2-day intervals. Test dose (1000 × 10^6 bacteria in volume of 1 c.c.) given intravenously 8 days after the last injection. The results are given in Table IX.

TABLE IX.

Vaccinated with—	Total number of rabbits.	Died during vaccination period.	Died after infection.	Survived.
P1	8	0	0	8
P2	5	2	2	1
C	5	1	2	2
Q	2	1	0	1
T	3	0	1	2
Unvaccinated	10	..	8	2

Experiment 2.—Immunization of rabbits with the atoxic fractions P1 and T alone and in combination with the toxic fraction P2. Each group of rabbits received 4 intravenous injections of antigen at 2-day intervals, 3 times

1 c.c. and then 2 c.c. Test dose as in Experiment 1. The results are given in Table X.

TABLE X.

Group.	Antigen received.	Total number of rabbits.	Died during vaccination period.	Died after infection.	Survived.
1	3 × 1 c.c., 1 × 2 c.c. of T	4	1	1	2
2	3 × 1 c.c., 1 × 2 c.c. of P1	3	0	0	3
3	3 × 1 c.c., 1 × 2 c.c. of P2	3	2	0	1
4	3 × 1 c.c. of T, then 2 c.c. of P2	3	0	0	3
5	3 × 1 c.c. of P1, then 2 c.c. of P2	3	0	0	3
6	Control, unvaccinated	2	..	2	0

Experiment 3.—Effect of purification (precipitation by acid) on the toxic and immunizing effect of P1 and P2. Method of immunization: 3 intravenous injections of antigen at 2-day intervals. Test dose as in Experiment 1. The results are shown in Table XI.

TABLE XI.

Vaccinated with—	Total amount of antigen.	Total number.	Died during vaccination.	Died after infection.	Survived.
P1 (crude)	3 × 0·5 c.c.	4	0	1	3
P1 (purified)	Ditto	3	0	0	3
P2 (crude)	,,	4	2	0	2
P2 (purified)	,,	3	1	0	2
P1 (crude)	3 × 2·0 c.c.	4	1	0	3
P1 (purified)	Ditto	4	2	0	2
P2 (crude)	,,	3	3	0	0
P2 (purified)	,,	3	3	0	0
Control, unvaccinated	..	4	..	4	0

DISCUSSION.

By extraction of *B. dysenteriæ* (Shiga) by different procedures it is possible to obtain substances of different antigenic and immunizing properties and of varying degrees of toxicity. The toxic fraction of the bacterial bodies is associated with the saline extract (P2), and can be precipitated from it with acetic acid. In this respect the results correspond with those reported by Neisser and Shiga (1903) and by Di Donna (1908). The substance obtained by extraction with 75 p.c. alcohol exerts a weak toxic effect, which is demonstrable only by the method of Shwartzman. None of the substances obtained by acid-alcohol extraction (Q, T), or by saline extraction of acid-treated bacteria (Gl, Sa) shows any toxic effect. The carbohydrate fractions (C, Ha) are also devoid of toxic properties.

The production of specific agglutinins seems to be associated with the alcohol-soluble substances (P1, Q, T). Of the carbohydrate fractions the C substance containing 3·7 p.c. of nitrogen produced a small quantity of agglutinin, while the hapten prepared according to White (1933) and containing only

0·8 p.c. of nitrogen was ineffective. It seems, therefore, that the short alkali treatment required for the preparation of the C fraction is not sufficient to produce a pure hapten, while the repeated alkali treatments required for the preparation of the biuret-negative hapten of White (1933) effects a complete haptenization.

Of considerable interest is the fact that the toxic fraction P2 produced only a small quantity of agglutinins. It seems that there is a distinction between the toxic and the agglutinin-producing properties of the different substances. These facts are of interest with regard to the relation of bacteriolysis to the neutralization of endotoxin. Recently Felix and Pitt (1934) suggested that the O antigen, which is responsible for the production of complement-fixing and bacteriolytic antibodies, may also be responsible for the production of anti-endotoxic immune bodies. This view is justified if the body substance of the bacterium is taken as a whole. Our results, however, indicate that it is possible to separate from the body-substance of the bacterium fractions with different toxic and antigenic properties, and that the atoxic components are much more active antigens for agglutinin-production than the toxic ones.

These results were supported by the neutralization and immunization experiments. The best neutralizing antisera for the toxic " injury-producing factors " (Shwartzman, 1929) were obtained with the toxic P2 fraction, whereas in the passive immunization experiments with rabbits the serum prepared with P1, which contained large quantities of agglutinins, showed the best protective effect.

Similar results were obtained with mice, but this experiment showed that the protective effect of the P1 serum may be strengthened by the addition of an equal quantity of P2 serum. It seems that for passive protection the optimal effect is obtained with a serum mixture containing both agglutinins and anti-endotoxic immune bodies.

Corresponding results were obtained by active immunization. Vaccination with P1 effected the best protection against an infection. Especially the antigen prepared from the crude extract showed a low toxicity and a high immunizing effect. Of 19 rabbits immunized with this antigen, only one died during the immunization period and only one died after infection with living bacilli. All the other antigens showed either a high toxicity as in the case of P2 or an irregular immunization effect, as in T and Q. In this respect our results are similar to those reported by White (1933), whose attempts to immunize rabbits with the Q and T fractions of a salmonella organism failed. However, Experiment 2 shows that T as well as P1 may produce immunity against the toxic fraction P2 ; they may, therefore, be employed for pre-immunization purposes if the toxic antigen P2 must be employed.

The question arises why the crude P1 fraction possesses these enhanced immunizing properties. Furth and Landsteiner (1928) showed that the P1 fraction derived from *B. typhosus* reacted weakly with an immune serum for the C fraction of that organism. Since this reaction did not disappear after treatment with antiformin, they concluded that the P1 fraction contained small amounts of C. The content of C in this preparation could be reduced by repeated precipitation with acid. In the case of *B. paratyphosus B* they also found that the P1 fraction contained protein as well as a considerable

amount of carbohydrate. Chemical analyses of our P1 fraction of *B. dysenteriæ* (Shiga) show a similar composition ; the crude extract contained only 2·3 p.c. nitrogen and, after hydrolysis with hydrochloric acid, showed the presence of reducing sugars. After repeated precipititations with acetic acid the nitrogen content rose to 7·3 p.c. and the reducing substances disappeared. It seems, therefore, that the immunizing effect of P1 (crude) is due to the fact that it contains also the specific carbohydrate in combination with a relatively atoxic protein.

The heterophilic antigen of *B. dysenteriæ* Shiga seems to be associated mainly with the P2 fraction.

SUMMARY.

(1) The endotoxic effect of *B. dysenteriæ* (Shiga) is associated mainly with the saline-soluble antigen P2.

(2) Acid treatment, as required for the preparation of fractions Q and T, reduces the toxicity of the bacterial bodies ; the acid-soluble substances Q and T as well as the saline extracts prepared after the acid treatment (Gl, Sa) are atoxic ; these fractions give irregular immunizing effects.

(3) The substance P1, as well as T, immunized against a subsequent injection of the toxic substance P2.

(4) Active immunization with crude P1, which contains the specific carbohydrates in combination with a relatively atoxic protein, gave the best protection against infection with living bacteria.

(5) Passive immunization with the agglutinating antiserum of P1 in combination with the endotoxin-neutralizing serum of P2 gave the best protection against an infection with living bacteria.

(6) The heterophilic antigen of *B. dysenteriæ* (Shiga) is mainly associated with the P2 fraction.

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A NOTE ON THE STABILITY OF THE *GRAVIS*, *MITIS*, AND INTERMEDIATE TYPES OF *C. DIPHTHERIÆ*.

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Received for publication October 20th, 1935.

THE following is a *résumé* of the results obtained in attempting to alter the type of certain strains of *C. diphtheriæ* by in-vivo and in-vitro methods.

Christison (1933) showed that by frequent subculture of *C. diphtheriæ* in broth considerable variation could be induced in the colony appearance of any given strain. She found that after 12 to 14 generations (cultured at 3-day intervals) *mitis* strains developed *gravis*-like colonies on McLeod's medium, and that colonies having a *mitis*-like appearance developed in cultures which had yielded formerly only *gravis* colonies. The alteration in colony appearance was not accompanied by any change in the fermentative reactions. This work has been repeated and confirmed by Robinson (1934), who also failed to demonstrate any alteration in the fermentative reactions. He succeeded in one case, however, by subculturing papilla-like outgrowths from the colonies of an intermediate strain in obtaining from it a starch-fermenting strain. McLeod (1933) records one *gravis* strain which lost its power to ferment starch after prolonged maintenance amongst the stock cultures ; it had not, however, been an absolutely typical *gravis* organism from the first.

A repetition of Christison's work by the writer gave essentially similar results. Not only were the fermentative reactions of the variant colonies found to be unaltered from those of the original strain, but in four instances "*gravis*-like" *mitis* and smooth-surfaced *gravis* colonies were identical in serological grouping with the strains from which they were derived. The maximum number of variant colonies appeared at the 12th and 14th subcultures and thereafter tended to diminish. Because of the ease with which variation in colony appearance could be induced, and because it is generally conceded that the starch-fermenting power of the *gravis* type is its most characteristic cultural quality, it was decided to concentrate on an effort to produce this power in a *mitis* strain and to deprive a *gravis* strain of its starch-fermenting ability.

In-vivo experiments.—Because of its relative insusceptibility to infection by *C. diphtheriæ* the mouse was chosen as the experimental animal.

(1) It was thought that serial passage through a relatively insusceptible host might induce a change in type. A *gravis* and a *mitis* strain were each carried through a series of 6 mice. The cultures obtained from the spleens of the animals were examined before inoculation into the next animal in the series. In neither series did any alteration in the fermentative or cultural characters occur.

(2) Perry (1933) had shown that after inoculation of a rough strain of *B. typhosus* in a dose just sufficient to cause death in a mouse after 24–48 hours it

was possible to culture from the heart blood of the animal a highly virulent smooth strain of *B. typhosus*.

It was thought that this method might enable one to transmute the relatively less pathogenic *mitis* type into the virulent *gravis* type of *C. diphtheriæ*. Previous experiment had shown that the fatal dose of *C. diphtheriæ* for a mouse (irrespective of whether it was of the *gravis*, *mitis* or intermediate type, and whether recently isolated or not) was about 4·5 thousand million of a 24-hour culture intraperitoneally. To 45 mice, therefore, were given 4·5 thousand million of a 24-hour culture suspended in saline. In no case was a change of type observed in the organisms cultured from the spleen or heart blood where positive cultures were obtained.

Over the whole course of the experiments 97 mice were inoculated with 49 strains of *C. diphtheriæ* (16 *gravis* strains, 17 *mitis* strains and 16 intermediate strains) in doses varying from 1 to 9 thousand million organisms. Fifty-three animals died after periods of 1 to 13 days. Cultures were made from the spleen and heart blood in each case, and also from the 44 surviving animals when they were killed off. In 13 animals positive cultures were obtained from both spleen and heart blood, but in 31 animals spleen culture alone was positive. In 53 cases no growth was obtained. None of the 44 positive cultures showed any change in type.

It was therefore decided to use animals which, normally susceptible to infection by *C. diphtheriæ*, had been immunized against such infection. Eight guinea-pigs were immunized by gradually increasing doses of dead *C. diphtheriæ* over a period of 8 weeks, the inoculations being intraperitoneal and at 5-day intervals. Two of the animals which had been immunized with a *mitis* strain were inoculated with a live culture of *gravis* organisms ; 2 which had been immunized with a *gravis* strain were inoculated with a live culture of *mitis* organisms ; and the other 4 animals were inoculated with live cultures of the strains with which they had been immunized (2 *gravis* and 2 *mitis*). Fresh guinea-pigs were used as controls. In each case the inoculation was a thousand million organisms of a 24-hour culture administered intraperitoneally in a bulk of 1 c.c. saline. Subcultures were made from the peritoneal fluid at 8-, 24- and 48-hour intervals. In every instance cultures of *C. diphtheriæ* were obtained after 8 hours, but at 24 hours only 5, and at 48 hours none, were positive. None of the organisms recovered showed any change in type.

Similar negative results were obtained when cultures of *C. diphtheriæ* were recovered from the local lesions of 2 rabbits (previously immunized) which had received an intracutaneous inoculation of *C. diphtheriæ* along with sufficient antitoxin to prevent a fatal result.

In-vitro experiments.—Gravis and *mitis* strains of *C. diphtheriæ* were subcultured at 3-day intervals in glucose broth containing 5 p.c. *mitis* and *gravis* antisera respectively. It was found that after 2 subcultures the *mitis* strains developed the power to ferment starch (in Hiss's serum peptone water medium), but that the *gravis* strains showed no change in their fermentative reactions. This acquired power to ferment starch, however, could only be demonstrated when the subculture was made directly from the broth into the starch medium. After one subculture on ordinary solid media the acquired power to ferment starch was lost.

As it was conceivable that the fermentation was due to the small amount of glucose carried over in the subculture it was discarded from the broth, but without in any way affecting the results. It was also found that the same result was obtained when the broth contained as little as 0·1 p.c. antiserum, and that it was immaterial whether the antiserum was specific for the strain, or even for the type, under investigation or not. After 4 subcultures normal rabbit serum (5 p.c. in broth) gave the same result. No method was found of stabilizing the acquired quality. After 20 subcultures at 3-day intervals the starch-fermenting ability of such *mitis* strains disappeared after one subculture on ordinary medium. Only in one instance was there an exception to this—a *mitis* strain continued to ferment starch after having been subcultured on serum agar, but after a second such subculture it lost the power to do so.

The quality of rabbit serum which induced the change was found to be unaffected by heating to 56° C. for 30 minutes.

An investigation into the power of other mammalian sera to produce the same effect showed that normal ox, human, sheep and horse sera were ineffective after 10 subcultures, while normal guinea-pig serum had the same effect as normal rabbit serum. Commercial antitoxin (as was to be expected in view of the results with horse serum) did not affect the power of *mitis* strains to ferment starch when added to the broth in which they were grown.

Intermediate strains of *C. diphtheriæ* also became capable of fermenting starch when grown under similar conditions to those described for the *mitis* strains.

SUMMARY.

Passage of *gravis*, *mitis* and intermediate types of *C. diphtheriæ* through mice failed to alter the fermentative characteristics of the organisms.

Inoculation of live cultures of *gravis*, *mitis* and intermediate types into immunized guinea-pigs and rabbits induced no change in their fermentative characteristics.

C. diphtheriæ subcultured at 3-day intervals in broth containing normal rabbit or normal guinea-pig serum induced non-starch-fermenting strains to ferment starch after 2 subcultures. Similar culture in broth containing either ox, horse, human or sheep serum had no such effect on the starch-fermenting power of the organism. It was not found possible to stabilize this acquired property.

I wish to acknowledge much helpful criticism and advice from Prof. Tulloch during the course of this investigation.

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AN IN-VITRO INVESTIGATION OF THE REACTION BETWEEN DIPHTHERIA TOXIN AND ANTITOXIN.

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Received for publication October 22nd, 1935.

One of the fundamental problems of immunochemistry which has received much attention in the past is that of the nature of the reaction between toxins and antitoxins. It was dealt with at considerable length by Ehrlich (1898), Arrhenius and Madsen (1902), Bordet (1902), Craw (1907), Von Krogh (1916), Henry (1922), Glenny, Pope and Waddington (1925), and Burnet (1931). From the work of these investigators two main theories developed, of which one ascribed the neutralization of toxin by antitoxin to chemical combination, and the other to adsorption ; later workers in an effort to reconcile the two theories have suggested that it is due to both combination and adsorption.

These theories were based upon results obtained from work on many antigens and antibodies, but diphtheria toxin and antitoxin have been used by many investigators, who have generally worked on the partial saturation of toxin by antitoxin, although Glenny, Hopkins and Pope (1924) give in-vivo measurements of the residual antitoxin after the addition of toxin (or toxoid) to excesses of antitoxin. The partial saturation of antitoxin by toxin appears to have received little attention, yet an outstanding problem confronting the theorists—the Danysz phenomenon—(Danysz, 1902) is an effect which is produced by the addition of toxin in two portions to antitoxin, and therefore involves the partial neutralization of antitoxin by toxin at one stage in the reaction.

The major part of these earlier investigations had been made before the discovery of the Ramon flocculation method (Ramon, 1922) for the in-vitro determination of diphtheria toxin and antitoxin ; all the measurements had been made *in vivo*, and little later work has been published on the partial saturation of toxin and antitoxin using in-vitro methods. We therefore attempted to use in-vitro measurements to investigate the partial neutralization of diphtheria toxin by antitoxin and *vice versa*. By working on the neutralization of antitoxin we hoped to find an explanation of the Danysz effect, which Schmidt (1927, 1928) had claimed was demonstrable *in vitro*. The results obtained from an extension of Schmidt's work we shall describe in a later paper.

In the present paper we show that :—

(1) There is evidence for the assumption that diphtheria antitoxic sera will combine with toxin according to the law of multiple proportions.

(2) In the first stable compound toxin and antitoxin are present in equivalent amounts as in Ramon's "indicating mixture", *i. e.* 1 Lf unit of toxin (T) and 1 flocculation unit of antitoxin (A), which may be represented as TA. In the second compound the proportion of antitoxin is doubled, *i. e.* it has the composition TA_2.

(3) A mixture having the composition TA will combine with one equivalent of antitoxin and give the second compound TA_2.

(4) If toxin be added to a mixture having the composition TA_2, then it rapidly combines with toxin until toxin and antitoxin are present in equivalent amounts, *i. e.* the mixture has the composition TA.

The first experiment was devised to investigate the partial neutralization of toxin (or toxoid) by antitoxin (and *vice versa*) by finding the amount of toxin (or toxoid) or antitoxin which could be demonstrated by flocculation in the filtrates of mixtures which originally contained excess of either component after the primary reaction was complete.

EXPERIMENTAL.

Materials.

All diphtheria toxins, toxoids and antitoxins used throughout this work were drawn from stock routine products, except that toxins were preserved with 1/10,000 merthiolate in place of the usual phenol. The antitoxins used in this work were obtained as concentrated antitoxic sera and were diluted to give convenient numbers of antitoxin units per ml., the dilution being made with sodium chloride solution, such that the final concentration of salt was 2 p.c.

We wish to thank Mr. Glenny for giving us suitable antitoxic sera; Mr. Pope for the toxins and toxoids used, and Dr. Linggood for preparing the concentrated toxoid by ultrafiltration.

Method.

The general methods described by Glenny and Okell (1925) were adhered to for the estimation of toxin and toxoids, but for the standardization of antitoxins the serum was measured with a modified micrometer syringe (Trevan, 1925), and titrated against 1 ml. of known toxin.

The antitoxic filtrates from mixtures were estimated by using 1 ml. quantities of filtrate and varying additions of toxin or toxoid, except when the "blend" method was employed. In agreement with other workers we have been unable to show that the results obtained by using constant antitoxin and varying volumes of toxin or toxoid differ from those obtained by the more usual method.

The toxin or toxoid and antitoxin used for the estimation was the same as used for the preparation of the mixtures.

Toxins and antitoxins used in the preparation of mixtures were tested against each other, by the Ramon flocculation method, a sufficient number of times to determine the equivalent volume to within $\pm$ 0·5 p.c.

Filtrates shown in the tables as containing 0 to 50 toxin or antitoxin units per ml. are correct to the nearest 0·5 unit ; those containing from 50 to 100 units per ml., correct to 1 unit ; and above this value, correct to the nearest 2 units.

The Partial and Over-Neutralization of Toxin by Antitoxin.

Increasing amounts of antitoxin were added to volumes of toxin, so that a series of mixtures was obtained ranging from 100 p.c. toxin to 100 p.c. antitoxin. The mixtures were shaken, placed in a water-bath at 40° for 10 hours,

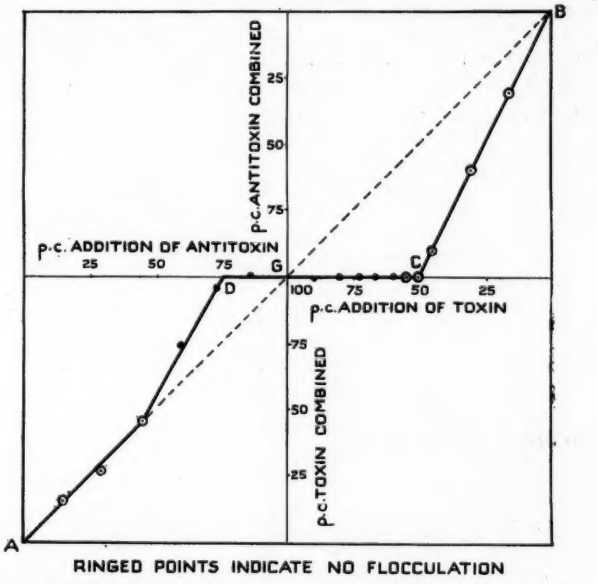

FIG. 1.—Result of partially and over-neutralizing toxin by antitoxin.

and left at room temperature for 2 days. They were then shaken again and filtered through a No. 5 Whatman filter paper, and the amount of toxin or antitoxin in the filtrate was determined.

The composition of the mixtures and the results are recorded in Table I. The columns are headed with the small letters (a)–(j), and in describing this table we refer to the columns by their letters. The composition of the mixtures by volume is shown in (a) and (b) ; and the total number of toxin and antitoxin units is given in (c) and (d). The number of toxin or antitoxin units per ml. of filtrate as estimated by the flocculation method is listed in (e) or (f), and in (g) and (h) the total number of units free in the mixture [calculated from (e) or (f)].

The values in (i) are calculated from the composition of the original mixtures ; when the toxin is in excess the number of antitoxin units added is expressed

TABLE I.

Materials.—Toxin TH 693, bottle A, 50 Lf units/ml. Antitoxin PC 2146, diluted to contain 390 flocculation units/ml.

(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)
Composition of mixtures.				Units found/ml. filtrate.		Total free units in mixture.		"Percentage addition" of toxin or antitoxin.	Percentage toxin or antitoxin combined.
Volumes.		Units.							
Ml. toxin.	Ml. antitoxin.	Toxin.	Antitoxin.	Toxin.	Antitoxin.	Toxin e (a + b).	Antitoxin f (a + b).	Antitoxin 100 d/c.	Toxin (c–g) 100/c.
100*	..	5,000	..	50·0	..	5,000	..	0	0
100*	1·92	5,000	749	41·0	..	4,179	..	15	16
100*	3·84	5,000	1,498	35·0	..	3,634	..	30	27
100*	5·76	5,000	2,247	26·0	..	2,750	..	45	45
100	7·68	5,000	2,995	12·0	..	1,292	..	60	74
100	9·12	5,000	3,657	4·5	..	491	..	73	96
100	9·60	5,000	3,744	0	..	0	..	76	100
100	10·88	5,000	4,243	0	..	0	..	86	100
100	12·82	5,000	5,000	0	..	0	..	100	100
								Toxin 100 c/d.	Antitoxin (d–h) 100/d.
135	19·20	6,750	7,488	..	0	..	0	90	100
120	19·20	6,000	7,488	..	0	..	0	80	100
110	19·20	5,500	7,488	..	0	..	0	73	100
100	19·20	5,000	7,488	..	0	..	0	67	100
90	19·20	4,500	7,488	..	0	..	0	60	100
110*	25·60	5,500	9,984	..	0	..	0	55	100
100*	25·60	5,000	9,984	..	0	..	0	50	100
90*	25·60	4,500	9,984	..	0	..	1,040	45	90
90*	38·40	4,500	14,976	..	47	..	6,035	30	60
90*	76·80	4,500	29,952	..	124	..	20,683	15	31
..*	1·00	..	390	..	390	..	390	0	0

* No flocculation.

as a percentage of the total number of toxin units (100 d/c); and when antitoxin is in excess the toxin added is expressed as a percentage of the antitoxin (100 c/d); we express this briefly as the "percentage addition" of toxin or antitoxin, as the case may be. The values in (j) are obtained, on the excess toxin side, by expressing the total number of toxin units combined as a percentage of the total number of units originally present in the mixture [*i. e.* (c–g) × 100/c]. On the antitoxin excess side the percentage of antitoxin combined will be given by (d–h) × 100/d.

An experiment was done using concentrated toxoid (210 Lf units/ml.) and antitoxin PC 2146. The results which are given in Table II show that concentration of the toxoid has no effect on the antitoxin-toxin combining ratio of the antitoxin.

TABLE II.

Materials.—Concentrated toxoid (210 units/ml.). Antitoxin PC 2146, diluted to contain 210 units/ml.

Composition of mixture.		Total antitoxin units (calculated).	Units free antitoxin per ml. (found).	Total free units antitoxin (found).	"Percentage addition" of toxoid.	Percentage. antitoxin combined.
Ml. toxoid.	Ml. antitoxin.					
5·00*	25	5250	104	3120	20	40·5
7·75*	25	5250	60	1965	31	62·0
10·00*	25	5250	31	1085	40	79·3
12·50	25	5250	0	0	50	100·0
17·50	25	5250	0	0	70	100·0
22·50	25	5250	0	0	90	100·0

* No flocculation.

In Fig. 1 we have plotted as ordinates the percentage of toxin or antitoxin combined (j, Table I), and as abscissæ the "percentage additions" of toxin or antitoxin (i, Table I). The line AGB would represent the theoretical neutralization of toxin by antitoxin (and *vice-versa*) if one unit antitoxin always combined with one unit of toxin. The point G represents a mixture containing equivalent amounts of toxin and antitoxin, *i. e.* it represents Ramon's "indicating mixture".

In the toxic mixtures which did not flocculate the percentage of toxin combined agreed very closely with the theoretical line AGB, which means that at that stage one unit of toxin was combined with one unit of antitoxin. In the flocculated mixtures, with increasing additions of antitoxin the amount of combined toxin rapidly approached 100 per cent., and when the "percentage addition" was 75 in this particular experiment (line DG in figure), all the toxin was apparently combined. Further additions of antitoxin must, therefore, give mixtures containing excess antitoxin, and the toxin present is then expressed as a "percentage addition" to the antitoxin.

On the excess antitoxin side free antitoxin could not be detected until the "percentage addition" of toxin is less than 50 (C). When the "percentage addition" of toxin is less than 50, all the values fall along the line CB, and therefore the ratio of percentage antitoxin combined to "percentage addition" of toxin is 2/1, *i. e.* when 1 unit of toxin is added to 2 units of antitoxin, the

whole of the antitoxin is apparently combined; we express this by saying that for values falling on the line CB the antitoxin/toxin combining ratio is 2/1.

We have repeated this experiment, using three other batches of toxin and two other antitoxins which had equal in-vivo and in-vitro values, and we obtained similar results. Sera which apparently have discrepant in-vivo and in-vitro values gave rather different results, which we discuss later.

The Addition of Toxin and Antitoxin to Neutral Toxin-Antitoxin Mixtures.

It seemed possible that the toxin-antitoxin complex forming in the presence of excess toxin or antitoxin had adsorbed or occluded the component in excess in the process of formation, and the following experiment was designed to see if a neutral mixture containing pre-formed floccules could remove toxin or antitoxin from solution.

A neutral toxin-antitoxin mixture was made by adding 500 ml. toxin TH 693 bottle B (51·5 Lf. units/ml.), to 49 ml. antitoxin PC 2146 (diluted to contain 525 flocculation units/ml.), and 24 ml. 1 p.c. saline, in order that the mixture might contain 45 toxin and antitoxin units/ml. After shaking, the mixture was placed in a water-bath at 40° C. for 4 hours, and was then kept at room temperature overnight. To 20 ml. portions of the well-shaken neutral mixture, varying amounts of toxin or antitoxin were added. After standing at room temperature for 2 days, the mixtures were filtered through No. 5 Whatman papers, and the free toxin or antitoxin determined in the filtrates. The results are given in Tables III and IV.

The composition of the final mixtures is shown in (a) and (b), and the total number of toxin or antitoxin units in the mixture is given in (c); this is the sum of the toxin units present in the neutral mixture, and the added toxin. The free toxin "estimated" per ml. of filtrate by the flocculation method is shown in (d), and the total free toxin in (e). In (f) the number of antitoxin units in the final mixture is expressed as a percentage of the total toxin present, and is headed "percentage addition antitoxin" because it is comparable to (i) in Table I—the only difference being that in the first experiment the final mixture was made in one stage, and in this experiment it was made in two stages, *i. e.* the preparation of the neutral mixture and the addition of toxin.

TABLE III.

Materials.—Toxin TH 693 (51·5 Lf units/ml.). Antitoxin PC 2146, diluted to contain 525 flocculation units/ml.

(a) Composition of mixture.		(b)	(c)	(d)	(e)	(f)	(g)
Ml. mixture.	Ml. toxin.	Total T units in mixture (calculated).	Units free T/ml. filtrate.	Total free T units in mixture.	"Percentage addition" of antitoxin.	Percentage toxin combined.
20	1·945	1000	0	0	90	100·0
20	4·370	1125	3	73	80	93·5
20	7·520	1285	10	257	70	78·6
20	11·700	1500	12	380	60	74·7
20	26·300	2250	25–26	1180	40	47·6
20*	70·000	4500	37–38	3375	20	25·0

* Floccules completely dissolved.

Table IV is similar to Table III except that columns (b), (c), (d), (e) and (g) refer to antitoxin and (f) to toxin.

TABLE IV.

(a) Composition of mixture. Ml. mixture.	(b) Composition of mixture. Ml. antitoxin.	(c) Total A units in mixture (calculated).	(d) Units free A/ml. filtrate.	(e) Total free A units in mixture.	(f) "Percentage addition" of toxin.	(g) Percentage antitoxin combined.
20	1·06	1125	0	0	80	100·0
20	2·85	1500	0	0	60	100·0
20	3·50	1635	0	0	55	100·0
20	4·29	1800	0	0	50	100·0
20	5·25	2000	0–1	12	45	99·4
20	6·43	2250	11	291	40	87·0
20	10·00	3000	36	1080	30	64·0
20	17·14	4500	66	2450	20	47·0
20*	37·60	9000	120	6912	10	22·0

* Floccules completely dissolved.

The results from Tables III and IV are plotted in Fig. 2 by the method employed for Fig. 1. The mixture of neutral floccules is indicated in the graph by the point G. We use the term "neutral floccules" to mean the product of combination of equal numbers of Lf units of toxin and flocculation units of antitoxin. Points to the right of G represent decreasing "percentage additions" of toxin, but, as pointed out in the description of the tables, this amounts to the increasing addition of antitoxin to the floccules. The points on the left-hand side of the graph are the result of excesses of toxin for the same reason. It will be seen that Fig. 2 is similar to Fig. 1.

If the neutral floccules neither adsorbed nor combined with toxin or antitoxin the results would fall along GA, GB. The lines GD, DA show that neutral floccules will adsorb or combine with a small amount of toxin. The line GC shows that for this particular experiment, neutral floccules will adsorb or combine with all added antitoxin until the "percentage addition" of toxin is less than 45 p.c. (*i. e.* the antitoxin-toxin ratio is 2·2 : 1), and below this value (the line CB) free antitoxin is present in the filtrate, and the values obtained show that the ratio of the percentage of combined antitoxin to the "percentage addition" of toxin tends to be slightly greater than 2 : 1.

We have repeated this experiment, using toxoid TMX 952 (46 Lf units/ml.) and toxin SBT 218 (31 Lf units/ml.), and have found that neutral floccules produced from them will adsorb antitoxin until the antitoxin-toxin ratio is a little greater than 2 : 1 ; we believe this adsorption to be due to the preformed floccules which are present in the majority of the mixtures, the floccules dissolving only in great excess of toxin or antitoxin. The amount of toxin or toxoid which can be adsorbed varies for different toxins, and does not seem to be directly dependent upon the mass of floccules.

From the results of these experiments we draw the following conclusions :

(1) By the direct addition of toxin or toxoid to antitoxin when the antitoxin is in great excess, one equivalent of toxin (as determined by the Ramon flocculation method) apparently removes two equivalents of antitoxin; and where the excess of antitoxin is less than two equivalents all the antitoxin present seems to be adsorbed or combined. We may express this approximately as—

$$T + 2A \rightarrow TA_2 \text{ and } Td + 2A \rightarrow TdA_2.$$

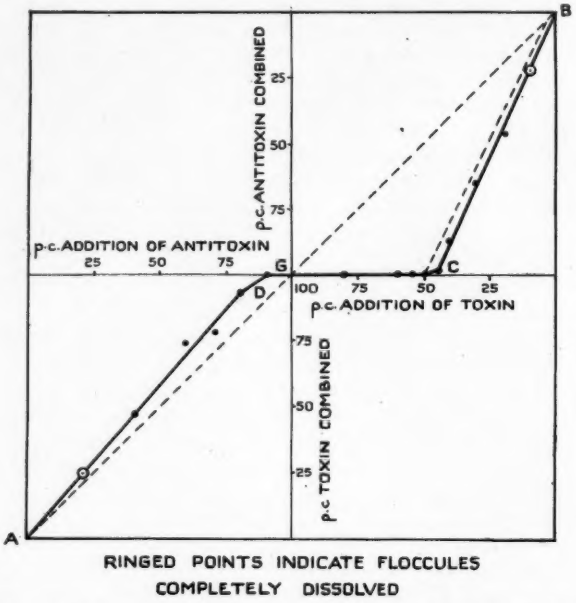

FIG. 2.—The addition of toxin and antitoxin to neutral toxin-antitoxin mixtures containing preformed floccules.

(2) Where the toxin or toxoid is in excess, fractional additions of antitoxin appear to remove equivalent amounts of toxin (within the limits of experimental error) in all mixtures that do not flocculate, and only where flocculation occurs are greater amounts removed. As shown in Table I, a 76 p.c. addition of antitoxin removes all the toxin, but this percentage varies with the toxin and antitoxin used.

(3) Neutral toxin-antitoxin (or toxoid-antitoxin) floccules will adsorb or combine with an amount of antitoxin that is slightly more than the antitoxin of the floccules, or expressed approximately:

$$TA + A \rightarrow TA_2.$$

(4) Neutral toxin-antitoxin floccules will combine with or adsorb small amounts of toxin or toxoid, but the amounts are not definitely related to the excesses of toxin or the amount of neutral floccules.

The Addition of Toxin and Antitoxin to Mixtures of One Equivalent of Toxin and Two of Antitoxin.

Since the complex TA_2 appears to be stable, it was of interest to see if it would combine with another equivalent of toxin to give 2TA.

A mixture containing one equivalent of toxin and two of antitoxin was made by adding 500 ml. toxin TH 693 (51·5 Lf units/ml.) to 98 ml. antitoxin PC 2146 (diluted to contain 525 flocculation units/ml.), and was left at room temperature for 2 days. To 20 ml. portions of the well-shaken mixture varying amounts of toxin and antitoxin were added as before, and, after standing for 48 hours at room temperature, the free toxin or antitoxin in the filtrate was estimated. The results are shown in Table V and Fig. 3. The table is similar to Tables III and IV, all the columns having the same significance.

TABLE V.

Materials.—Toxin TH 693, bottle B (51·5 Lf units/ml.). Antitoxin PC 2146, diluted to contain 525 flocculation units/ml.

(a)	(b)	(c)	(d)	(e)	(f)	(g)
Composition of mixture.		Total A units in mixture.	Units free A/ml. filtrate.	Total free A units in mixture.	"Percentage addition" of toxin.	Percentage antitoxin combined.
Ml. mixture.	Ml. toxin.					
20	16·70	1707	0	0	100	100·0
20	10·05	1376	0	0	80	100·0
20	2·96	1012	0	0	60	100·0
20	1·67	946	0	0	55	100·0
20	0	860	0	0	50	100·0

Composition of mixture.		Total T units in mixture.	Units free T/ml. filtrate.	Total free T units in mixture.	"Percentage addition" of antitoxin.	Percentage toxin combined.
Ml. mixture.	Ml. toxin.					
20	20·43	1910	0	0	90	100·0
20	25·10	2130	6	271	80	87·3
20	31·10	2460	11–12	562	70	77·0
20	39·10	2910	15	886	60	69·5
10	33·45	2150	25	1086	40	49·6
5*	37·60	2145	39	1661	20	22·0

Composition of mixture.		Total A units in mixture.	Units free A/ml. filtrate.	Total free A units in mixture.	"Percentage addition" of toxin.	Percentage antitoxin combined.
Ml. mixture.	Ml. antitoxin.					
20	1·91	2121	2·5	55	45	96·0
20	2·05	2150	5·0	110	40	95·0
20	5·46	2888	33·0	845	30	70·6
20	12·14	3777	49·0	1576	20	41·4
20*	32·76	8590	113·0	5962	10	21·0

* Floccules completely dissolved.

It will be seen that Fig. 3 is similar to Figs. 1 and 2. The composition of the original mixture is represented by the point C. If the floccules did not adsorb or combine with toxin the results would fall along CE, but the line CG shows that they will combine with all added toxin until there is just over one equivalent of toxin present for every equivalent of antitoxin (point D); beyond this value free toxin is found in the filtrates and the results fall along the line DA. The line OA represents the values which would be found if the floccules adsorbed or combined with exactly one equivalent of toxin. The line CFB shows that the floccules adsorb a very small amount of antitoxin.

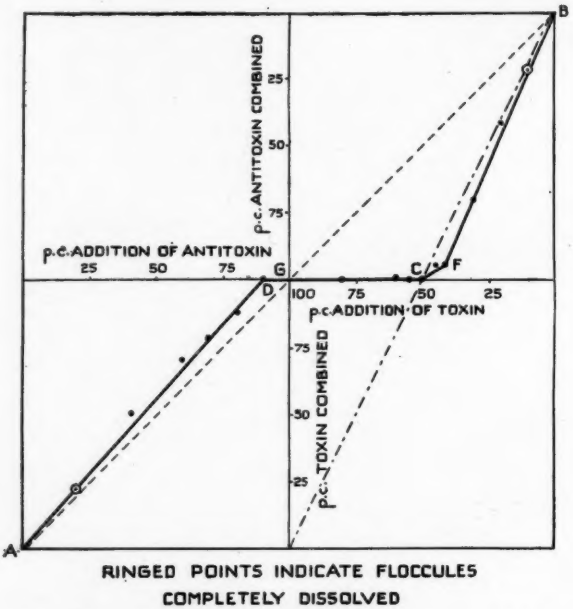

FIG. 3.—The addition of toxin and antitoxin to mixtures containing one equivalent of toxin and two equivalents of antitoxin.

From these results we draw the following conclusions:

(1) That floccules having the composition TA_2 will adsorb or combine with approximately one equivalent of toxin, *i. e.*

$$TA_2 + T \rightarrow 2TA$$

(2) That TA_2 floccules will adsorb only a very small amount of antitoxin.

The Antitoxin-Toxin Combining Ratios of Sera having Varying In-vivo/In-vitro Ratios.

As most of the preceding work was done with antitoxin PC 2146, which has an in-vivo/in-vitro ratio of 1·14, it might be argued that the formation of TA_2

was peculiar to that particular serum, therefore eleven other antitoxins of widely varying in-vivo/in-vitro ratios were tested.

To six known volumes of a serum varying amounts of toxin were added ; as before we have expressed the toxin units added as a percentage of the antitoxin

TABLE VI.

Serum No.	Antitoxin value. *In vitro.*	Antitoxin value. *In vivo.*	Ratio.	"Percentage addition" of toxin.	Percentage antitoxin combined.
12505	700	1300	0·540	15	21·5
				25	30·0
				35	43·5
				50	91·0
				70	?
				80	?
12507	600	800	0·750	10	15·4
				15	24·0
				30	49·0
				50	79·0
				55	84·0
				65	92·6
PK 2175	1450	750	0·830	20	35·0
				25	49·2
				30	59·5
				35	62·0
				40	72·8
				45	94·6
				50	100·0
				65	100·0
				85	100·0
D 1880	550	645	0·855	15	33·3
				25	52·6
				40	81·2
				50	97·3
				70	100·0
				90	100·0
PK 2014	1600	1700	0·960	15	25·0
				25	44·0
				35	70·0
				50	100·0
				65	100·0
				75	100·0
P 337	1300	1700	0·760	20	34·0
				30	57·6
				40	76·2
				50	100·0
				70	100·0
				90	100·0

TABLE VII.

No.	Antitoxin value. *In vivo.*	Antitoxin value. *In vitro.*	Ratio.	"Percentage addition" of toxin.	Percentage antitoxin combined.
PR 2172	1200	1200	1·00	20	40·4
				30	62·4
				40	80·0
				50	100·0
				70	100·0
				90	100·0
PK 2107	1400	1400	1·00	20	39·6
				30	61·6
				40	80·0
				50	100·0
				70	100·0
				90	100·0
PC 2146	600	525	1·14	20	41·3
				30	60·0
				40	80·0
				50	100·0
				60	100·0
				70	100·0
				80	100·0
				90	100·0
PR 2145	1100	900	1·22	20	39·0
				30	62·0
				50	100·0
				80	100·0
				95	100·0
D 1887	900	700	1·28	25	50·0
				35	69·5
				45	87·2
				50	96·0
				75	94·0
				85	90·0
12522	600	400	1·50	15	31·5
				25	51·2
				40	74·6
				50	100·0
				75	100·0
				95	100·0

units and have called this the "percentage addition of toxin". The mixtures were then either left for 12 hours at 45° and 12 hours at room temperature or for three days at room temperature. They were then filtered through No. 5 Whatman papers and the free antitoxin determined in the filtrate by the flocculation method. The results are given in Tables VI and VII and Figs. 4 and 5. In these figures the line GCB is the theoretical line

obtained on the assumption that one equivalent of toxin will combine with two equivalents of antitoxin.

It will be seen in Fig. 4 that sera with ratios less than unity do not show consistent antitoxin-toxin combining ratios of 2, and that the lower the ratio the greater the divergency from the line GCB ; but even with a ratio of 0·54 (12505), the 50 p.c. addition of toxin combines with 91 p.c. antitoxin, which closely approaches an antitoxin-toxin combining ratio of 2. We are investigating further the " low ratio " sera which show these discrepancies. From

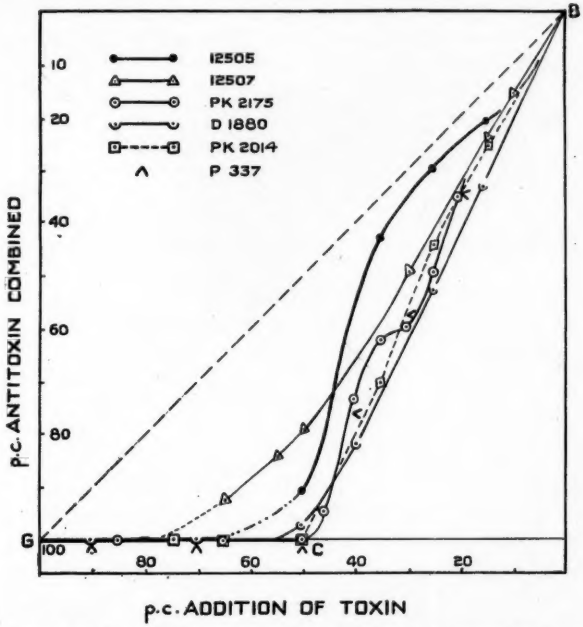

FIG. 4.—Variation of percentage of antitoxin combined, with increasing percentage additions of toxin, for six sera having in-vitro/in-vivo ratios of less than unity.

Table VII it is seen that sera having ratios of 1 or more have an antitoxin/toxin combining ratio of 2, except for one unconcentrated serum (D 1887).

The Solubility of Floccules.

The mixtures in which flocculation did not occur or the floccules dissolved have been indicated by asterisks in the tables, and in Fig. 1 it will be seen that the values for mixtures outside the limit of flocculation on the side of excess toxin fall on the line AG. The values of non-flocculating mixtures on the side of excess antitoxin fall on the line BC, which represents theoretical values for an antitoxin/toxin combining ratio of 2. In Figs. 2 and 3 it will be seen that as the floccules dissolve in the increasing excess of toxin and antitoxin, so the values approach the lines AG, BC.

It is known that flocculation occurs only in diphtheria toxin mixtures which contain approximately between $\frac{1}{2}$ to 2 equivalents of antitoxin. Marrack and Smith (1930) stated that "from mixtures containing excess of toxin or antitoxin precipitates are formed containing excess of one of these constituents". They published determinations of nitrogen content showing that as the antitoxin/toxin ratio increases, the amount of precipitate increases; but as their toxin would not flocculate when the antitoxin/toxin ratio was greater than 1·6, no values could be obtained above this value. When working with toxins which still flocculate when the antitoxin/toxin ratio is a little greater than 2,

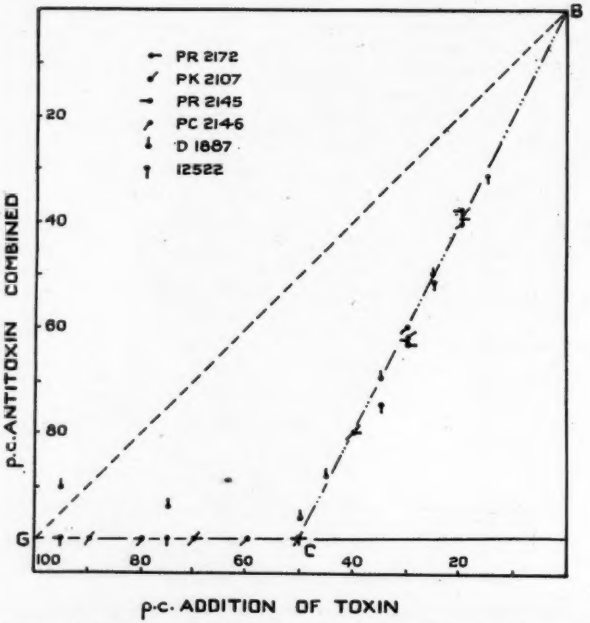

FIG. 5.—Variation of percentage of antitoxin combined, with increasing percentage additions of toxin, for six sera having in-vivo in-vitro ratios of equal to, or greater than unity.

we have found that the mass of floccules is at a maximum when there is approximately two equivalents of antitoxin, and above this level the mass rapidly decreases.

It has been observed in this work that preformed floccules are soluble in excess toxin, though complete solution is not obtained until the mixture has an antitoxin/toxin ratio of approximately $\frac{1}{3}$. Preformed floccules increase in mass on the addition of antitoxin until the antitoxin/toxin ratio is approximately 2/1; in greater excesses of antitoxin the floccules readily dissolve, and are almost completely in solution when the ratio is 2·5/1.

The following figures on the nitrogen content of floccules (Table VIII) show the validity of the above statement. Varying amounts of antitoxin were

added to 10 ml. quantities of toxin so that the toxin became different "percentage additions" to the antitoxin. The nitrogen content of the floccules thus produced was determined by the micro-Kjeldahl method. Materials used were toxin TH 688 and antitoxin PC 2146. The mixtures were made in 40 ml. centrifuge tubes, and after being stirred thoroughly were placed in a water-bath for 5 hours, when they were presumed to have flocculated completely. They were then centrifuged, the supernatants removed, and 25 ml. of 1 p.c. sodium chloride solution added to each tube. After being centrifuged, the supernatant was removed and the nitrogen determined in the floccules. See Table VIII (b).

A further experiment was done using neutral floccules as the starting material. Varying amounts of antitoxin were added to quantities of neutral floccules, each of which had been prepared from 10 ml. volumes of toxin. After a few hours the mixtures were centrifuged and dealt with in a manner similar to that in the previous experiment. Materials used were toxoid TMX 973 and antitoxin PC 2146. See Table VIII (c).

The experiments have been repeated a number of times and both series are typical examples. The results are shown graphically in Fig. 6.

TABLE VIII.

Materials.—Toxin TH 688 (42 Lf units/ml.). Toxoid TMX 973 (50 Lf units/ml.). Antitoxin PC 2146 diluted to contain 210 flocculation units per ml. The nitrogen content is given in mg. per 10 ml. toxin.

(a) "Percentage addition" of toxin.	(b) Mg. N.	(c) Mg. N.
100·0 (TA)	1·164	1·359
80·0	1·516	1·705
66·7	..	1·806
57·2	1·752	2·031
50·0 (TA_2)	1·814	2·217
44·5	1·610	..
40·0	0·713	2·025
33·0	..	1·306
25·0	..	0·102

The Danysz Phenomenon.

The fact that one equivalent of a toxin can combine with two equivalents of antitoxin to give a relatively stable complex may be a cause of the Danysz phenomenon.

Thus if to 100 units of antitoxin 40 units of toxin are added, 80 units of antitoxin would unite with the toxin; and when the second portion of toxin (60 units) is put in, 20 units would combine with the free antitoxin and there would be apparently 40 units toxin in excess. The composition of the mixture immediately after the addition of the second toxin fraction would be [40 (TA_2) + 20TA + 40T], and an instantaneous estimation would show the presence of 40 units of free toxin, and if it did not disappear this would cause the Danysz

phenomenon. But we have shown that free toxin can decompose the TA_2 complex, therefore after a time we should expect to find that the mixture was neutral, because 40 (TA_2) + 40T → 80TA. Arrhenius, working with tetanolysin, stated: "The strongly toxic solutions in which tetanolysin has been added in fractions slowly lose their abnormal toxicity, and after a time (6 hours at 37° C.) they are no more toxic than corresponding mixtures which have not been fractionated." Referring to this question Heidelberger (1933) said: "But this is exactly what would be predicted if toxin and antitoxin could combine, as Danysz maintained, in multiple proportions, and if the equilibrium at the equivalence point were reversible as Arrhenius believed, as we have shown to be the case for the analogous precipitin reaction."

An experiment was therefore designed to demonstrate this reversibility.

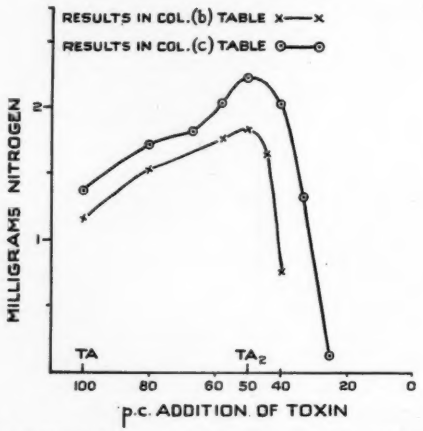

FIG. 6.—The relation between the composition of toxin-antitoxin floccules, and their nitrogen content.

The amount of toxin TH 688 equivalent to 12 ml. antitoxin PC 2146 was determined by the flocculation method, and 10–90 p.c. of this amount was added to each of nine bottles containing 12 ml. antitoxin. After being shaken these were left for 24 hours at room temperature. Portions of the well-shaken mixtures were then filtered and the free antitoxin in the filtrates was determined. A measured volume of the well-shaken mixture was taken and the required amount of toxin added, so that the total toxin content was then equivalent to the antitoxin content. Immediately after the addition of this second fraction portions of the well-shaken mixtures were filtered and the free toxin in the filtrates was estimated. After three days the remainder of the mixtures was filtered and the toxin or antitoxin content of the filtrates was again estimated. See Table IX.

The results confirm the argument expressed in the last section. It is obvious from the table that the abnormal toxicity induced by the fractional addition of an equivalent of toxin to antitoxin is a transitory phase in the

reaction $TA_2 + T \rightarrow 2TA$. This reaction must be fairly rapid, as the average time between adding the second fraction and putting up the test was about five minutes, and in each case less than the expected toxin value was found.

TABLE IX.

Materials.—Toxin TH 688 (42 Lf units/ml.). Antitoxin PC 2146, diluted to contain 525 flocculation units/ml.

Composition of mixture.		1st fraction.		2nd fraction.	
Ml. antitoxin.	Ml. toxin.	A units/ml. (calculated).*	A units/ml. (found).	T units/ml. found immediately.	T units/ml. (3 days).
12	15	187·0	186	2(4)	0
12	30	90·0	88	4(8)	0
12	45	44·0	44	9(12)	0
12	60	17·5	17–18	14(16)	0
12	75	0	0	7(20)	0
12	90	0	0	5(16)	0
12	105	0	0	0(12)	0
12	120	0	0	0(8)	0
12	135	0	0	0(4)	0

* Calculated on the assumption that one equivalent of toxin will combine with two equivalents of antitoxin. The bracketed figures give theoretical toxin values, assuming no reversal of TA_2.

DISCUSSION.

We have shown in this paper that there exists a complex of diphtheria toxin and antitoxin the composition of which appears to be two equivalents of antitoxin to one of toxin (TA_2). We have not yet found any record of a previous observation of this, although Heidelberger and Kendall (1929 and 1935) showed that the pneumococcus specific polysaccharide Type III and its homologous antibody combine in multiple proportions.

Throughout these experiments we have made the assumption that the first flocculating mixture in a Ramon test indicated the presence, in that mixture, of equivalent numbers of free toxin and antitoxin units. We have shown that the complex TA_2 can be decomposed by addition of toxin, but in testing mixtures which did not originally flocculate, and therefore contained the toxin-antitoxin complex in solution, the amount of free antitoxin as estimated by the Ramon method shows the formation of TA_2 in the original mixture. This indicates that the rate of decomposition of the complex by toxin is much slower than the rate of reaction between toxin and free antitoxin, which determines the first flocculating mixture in a Ramon range.

We have found certain sera which do not yield a complex of composition exactly TA_2, and it has been observed that there seems to be a correlation between the in-vivo/in-vitro ratio and the production of a "stable" TA_2 complex. A complex of this composition occurs consistently when the ratio is 1·0 or more, but when the ratio is less than 1·0 the antitoxin combined is always less than two equivalents, and although the amount combined may be inconsistent, yet, on the whole, it approaches more closely to two equivalents than one.

In referring to the complex and its formation we have used the term "adsorb or combine with antitoxin" because we have not done extensive experiments to prove whether it is true chemical combination or adsorption, but for the following reasons we believe that it is not adsorption :—

(1) The values obtained are not in accordance with those required by Freundlich's isotherm, as obviously the amount of antitoxin adsorbed does not increase with increasing concentrations of antitoxin.

(2) Neutral floccules consistently remove one other equivalent of antitoxin, independent of conditions.

(3) The fact that the antitoxin/toxin combining ratio in presence of excess antitoxin is 2 suggests chemical combination in multiple proportions.

As shown in the figures, neutral floccules adsorb or combine with small amounts of toxin, but since this amount varied considerably and inconsistently with the toxins and antitoxins used, we assume that this is not chemical combination, but adsorption on to the floccules present. For this same reason we think the small amount of antitoxin taken up by TA_2 floccules (as in Figs. 3 and 4) making the antitoxin/toxin ratio greater than 2/1, is adsorption.

Though we have written the equation $TA + A \rightarrow TA_2$ we suggest, not that it is irreversible, but that we have no means of showing its reversibility ; the equation $TA + A \rightleftharpoons TA_2$ is probably a truer representation, but the ratio $[TA][A] / [TA_2]$ must be very small, and our experimental technique precludes our measuring less than half a flocculation unit of antitoxin. Arrhenius and Madsen (1909) claimed that $[T][A] \rightleftharpoons K[TA]^2$, but any attempt to measure this alleged free toxin and antitoxin by the flocculation method must of necessity fail, because it involves the addition of free antitoxin or toxin, which would upset the equilibrium.

There is no evidence of the existence of complexes of higher antitoxin content (*e. g.* TA_3), and if such complexes exist they must be so unstable that, on the addition of toxin, the rate of reversal is so rapid that their presence does not effect the flocculation in the Ramon method.

It is probable that there are two contributory causes to the Danysz effect. The formation of TA_2 by the first toxin fraction and the subsequent addition of the second fraction would give a highly toxic effect, which would disappear in a relatively short time. Thus we have shown that filtrates which were abnormally toxic immediately after the addition of the second toxin fraction contained no toxin when tested after 4 days. It seems from this evidence that any permanent Danysz effect must be due to the toxoid content of the toxin used (Glenny, 1931), and the use of in-vivo methods for the determination of the antitoxin equivalent of the toxin-toxoid mixture. A toxin devoid of toxoid would not be expected to give a permanent Danysz effect, nor would toxin containing toxoid if the antitoxin equivalent were determined by the flocculation method and not the in-vivo method.

SUMMARY.

It is shown by in-vitro methods that :

1. Toxin or toxoid readily combines with approximately two equivalents of antitoxin in the presence of excess antitoxin (TA_2).

2. A mixture containing equivalent amounts of toxin and antitoxin (TA) will readily combine with approximately one further equivalent of antitoxin (TA_2).

3. This complex combines with one equivalent of toxin or toxoid to give a compound equal in all respects to that produced by the addition of one equivalent of antitoxin to one of toxin (TA).

4. The small amount of toxin or toxoid which is adsorbed by neutral floccules varies with conditions, and there is no evidence of the formation of T_2A.

5. The Danysz phenomenon cannot be wholly explained by these facts.

6. Determinations of nitrogen content are given which show that the mass of floccules increases as the composition changes from TA to TA_2.

We wish to express our grateful thanks to Mr. C. G. Pope for his help and encouragement throughout this work, to Dr. J. W. Trevan for his interest and his constructive criticism during the preparation of the manuscript, and to E. J. Knell for doing the micro-nitrogen determinations.

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OPTIMAL PRECIPITIN REACTIONS.

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Received for publication October 22nd, 1935.

THE precipitate which appears when an antigen is mixed with its antiserum in suitable proportions has been used frequently for the quantitative determination of antibodies and antigens. Thus, the specific optimal precipitation between diphtheria toxin and its antitoxin was made the basis of a method for the quantitative estimation of either the antigen or antitoxin (Ramon, 1922); for the estimation of tetanus toxin and antitoxin (Descombey, 1924); and of staphylococcus toxin and antitoxin (Burnet, 1931). Early workers with antisera to common protein expressed their results in the form of a dilution figure, *i. e.* the highest dilution of the antiserum which gave a precipitate with the antigen under certain prescribed conditions, whereas for bacterial toxins the value is determined by the mixture of toxin and antitoxin which flocculates more rapidly than any other mixture. A similar quantitative aspect was introduced into ordinary precipitin work by Opie (1923), and Dean and Webb (1926), who showed that if sufficiently dilute antibody solutions were used, and the antigen concentrations were varied, optimal flocculating zones could be obtained, similar to those observed in the Ramon reaction. Morgan (1923) also found optimal zones with the reaction between pneumococcus soluble-substance and antiserum. Generally, the supernatant solution from the Ramon "optimal flocculating mixture" contains no excess toxin or antitoxin, and similarly the supernatant from the Dean and Webb optimal flocculating mixture contains no excess antigen or antibody.

Marrack (1934), in his monograph on the chemistry of antigens and antibodies, has reviewed the work on precipitins, and has used the term "procedure α" for the constant antibody method introduced by Dean and Webb, and "procedure β" for the constant antigen method commonly used for the flocculation of toxins and antitoxins. For brevity, these terms will be used in this paper. All workers with the pneumococcus specific carbohydrate as antigen have found that whereas the α procedure gave neutral flocculation, the reverse β procedure only gave flocculations with very large amounts of antibody and these obviously were not neutral. Duncan (1932) obtained similar results using yeast gum and various bacteria as antigens; two distinct optimal zones depending on the method used (α and β) were obtained. In 1934 he extended his work using *Bact. dysenteriæ* Flexner "Z".

The writer has also found two optimal zones of flocculation between Type I pneumococcus antisera and its specific carbohydrate, depending on the method used, and it was the investigation of these two zones which led to the work on precipitins described in this paper.

It has been found that precipitin antigen-antibody reactions may be grouped into three classes as follows :

1. Those antigens and their antibodies in which both the α and the β procedures give the same serum antigen/antibody ratio.

2. Those in which the α and β methods give different antigen-antibody ratios, but in which the β method by the treatment described in this paper can be made to give the same ratio as the α method.

3. Those in which the α or β methods give different antigen-antibody ratios, and in which treatment of the β method as in (2) fails to bring about a coincidence of the ratio.

METHODS.

Throughout the work the technique described by Wilson Smith (1932) has been followed whenever the volumes to be dealt with made it possible, but with some of the antisera which had a low titre volumes in excess of 1 ml. were required, and these additions were made with pipettes ; the necessary degree of mixing was obtained by capping each tube with a small square of paper and inverting. As Wilson Smith points out, the time lag between mixing the contents of each tube is the factor which most readily produces unreliable results. When the necessary care is taken, I have found the results obtained by the pipette technique agree to within 20 p.c. with the values for the Wilson Smith syringe method. Unless otherwise stated, all mixtures were incubated in a constant-temperature bath at 40° C., two-thirds immersed to produce the necessary convection currents.

OPTIMAL PRECIPITIN REACTIONS WITH CONSTANT ANTIBODY CONCENTRATIONS AND VARYING ANTIGEN CONCENTRATIONS, AS COMPARED WITH THOSE WITH CONSTANT ANTIGEN CONCENTRATION AND VARYING ANTIBODY CONCENTRATIONS.

Reactions between Types I and II Antipneumococcus Serum and their Respective Soluble Specific Substances (S.S.S.).

Optimal flocculating values were determined for a number of Type I antisera with isotonic borate buffer solution for the S.S.S. solvent and 1 p.c. saline for the serum diluent as recommended by Wilson Smith, and varying the concentration of the S.S.S. (α method). The same sera were then titrated by the reverse β method in order to determine whether there was a constant relationship between the values obtained by the α and β methods respectively. In the determination of the β values the concentration of antigen used was that present in the optimal mixture found for the α method, and a wide range of antibody concentrations was put up against this. The β titres were readily obtained, but no relationship appeared to exist between the α and β values, as is shown in Table I.

Dilution of the antibody with non-specific serum instead of with 1 p.c. saline produced no observed change in the position of the β optimum, as would have been expected had the production of this zone been due to the physical influence of excess protein.

TABLE I.—*Showing the Ratios between the α and β Flocculating Zones for Antipneumococcus Serum and Soluble Specific Substances.*

Antisera.	Protective titre : British units.	S.S.S. = 1 ml. antisera. α.	S.S.S. = 1 ml. antisera. β.	Ratio $\frac{\alpha}{\beta}$.
A.14577	50	$1{\cdot}0 \times 10^{-4}$	$0{\cdot}59 \times 10^{-4}$	1·7
A.14984	300	$1{\cdot}2 \times 10^{-4}$	$0{\cdot}34 \times 10^{-4}$	3·6
A.14761	500	$3{\cdot}5 \times 10^{-4}$	$0{\cdot}10 \times 10^{-4}$	35·0
NP.46	500	$1{\cdot}1 \times 10^{-4}$	$0{\cdot}05 \times 10^{-4}$	20·0
NP.72	800	$1{\cdot}1 \times 10^{-4}$	$0{\cdot}05 \times 10^{-4}$	20·0

The results are given in grams of a stock S.S.S. which had been purified to give flocculation at a dilution of 1 in 1,000,000 with antiserum.

It will be noticed that the protective titres of the sera given in Table I do not show a constant ratio to their precipitin titres. Wilson Smith, Fischer and Llewellyn Smith (1934) also point out that some sera as well as concentrated sera have precipitin titres which are not in agreement with their protective titres. These sera are probably exceptional, as extensive tests on experimental bleedings from three horses during nine months (first immunization) revealed that the precipitin titres of two were in good agreement with the protective titres, while that of the third was very much lower.

During the work on the titration of pneumococcus antiserum by the β procedure the following interesting observations were made. A series of tubes containing mixtures with constant antigen (S.S.S.) and varying antibody concentrations were left in the constant-temperature bath at 40° C. after reading the β optimal zone, until flocculation in every tube was complete—a matter of 3 or 4 hours. Each tube was then inverted twice and the series examined. Certain of the floccules had resisted dispersion or were stable; these tubes were found to correspond to the α optimal mixtures. In Table II the results are given for two sera treated in this way. If we consider serum H.4817, which is typical of many, titrated against a 1 in 100,000 dilution of S.S.S., the β optimal zone occurred at 1 in 5 dilution of serum. After the mixtures had remained in the bath for 3 to 4 hours, complete flocculation had occurred with every dilution from 1 in 1 to 1 in 50. The tubes were then inverted twice, and it was then found that at dilution 1 in 1, 1 in 2, 1 in 5 and 1 in 50 the flocculated material dispersed completely. At a dilution of 1 in 10 there was a slight dispersion, while at 1 in 20 dilution the inversion had no effect on the floccules. This dilution of serum and antigen was the α optimal mixture. It is proposed in this paper to refer to the modification of the β procedure as the γ method. It will be seen also from Table II that whereas with the γ method the serum dilution varied with the S.S.S. concentration, the β method gave optimal flocculation with a serum dilution of 1 in 5 for S.S.S. in concentrations of 1 in 50,000, 1 in 100,000 and 1 in 200,000. Serum A.14984 is unusual; the β optimal zones moved to increased serum dilution as the S.S.S. was diluted, so that the β and γ results are related in a manner similar to that described by Duncan (1934) for optimal A and B zones of the agglutination of certain bacteria. It is interesting that when Duncan's A and B series are dispersed and allowed to

agglutinate again, it is the zone which we have described as the β zone which is stable. With pneumococcus S.S.S. floccules, whichever method, the α or β, is used, it is the zone about the α optimal mixture which is stable.

A wide range of serum dilutions as shown in Table II is, of course, an orientating range, and when the tests were put up at 20 p.c. differences in serum concentration, the range covered was not always sufficient to give

TABLE II.—*Showing β Optimal Zones and the Position of the most Stable Floccules after Inversion of Tubes. β Optimum* 1. *Stable Floccules* 1.

Serum No.	S.S.S. dilutions.	Serum dilutions. 1/1.	1/2.	1/5.	1/10.	1/20.	1/50.
H.4817 bled 17.vii.34	1/50,000		2	1	3		
				3	1	*2*	
	1/100,000	3	2	1	3		
					2	1	*3*
	1/200,000	3	2	1	3		
						1	1
A.14984	1/50,000	1	1				
			3	1	*2*		
	1/100,000	3	1	1			
				2	1	*3*	
	1/200,000		3	1	1		
					2	1	*3*

Order of flocculation = 1, 2, 3. Order of stability = *1, 2, 3.*
Suspension of maximum stability corresponding to value 1.

TABLE III.—*Showing the Soluble Specific Substance Equivalent of a Number of Type I and Type II Antisera Determined by the α Constant Antibody Method and by the γ Constant Antigen Method.*

Antisera.	S.S.S. = 1 ml. serum : α.	S.S.S. = 1 ml. serum : γ.
Type I.		
A.14984	$1{\cdot}20 \times 10^{-4}$	$1{\cdot}37 \times 10^{-4}$
A.16391	$3{\cdot}28 \times 10^{-4}$	$3{\cdot}50 \times 10^{-4}$
50 p.c. A.14984 + 50 p.c. A.16391	$2{\cdot}13 \times 10^{-4}$	$2{\cdot}28 \times 10^{-4}$
H.4543	$0{\cdot}15 \times 10^{-4}$	$0{\cdot}10 \times 10^{-4}$
NP.118	$1{\cdot}15 \times 10^{-4}$	$1{\cdot}03 \times 10^{-4}$
NP—119	$2{\cdot}13 \times 10^{-4}$	$1{\cdot}95 \times 10^{-4}$
NP.121	$1{\cdot}94 \times 10^{-4}$	$2{\cdot}05 \times 10^{-4}$
NP.124	$0{\cdot}92 \times 10^{-4}$	$1{\cdot}08 \times 10^{-4}$
Type II.		
H.4384	$6{\cdot}80 \times 10^{-2}$	$6{\cdot}53 \times 10^{-2}$
H.4205	$5{\cdot}10 \times 10^{-2}$	$5{\cdot}70 \times 10^{-2}$
NC.186	$69{\cdot}00 \times 10^{-2}$	$73{\cdot}00 \times 10^{-2}$
NC.188	$51{\cdot}00 \times 10^{-2}$	$49{\cdot}00 \times 10^{-2}$

mixtures which showed complete dispersion on inversion of the tubes. Nevertheless, provided the tests were on the correct range, one, or at the most, two of the mixtures contained floccules which were more stable than those in the other mixtures. In some cases readings could be taken more easily after the mixtures had been in the water-bath at 40° C. overnight. Table III shows values obtained for a number of sera by the α method in parallel with the values obtained by the γ method. Both concentrated and unconcentrated sera were used, and although the floccules given by the concentrated sera were more readily dispersed, the agreement in both between the α and γ methods was good. This reversal of the pneumococcus antibody and S.S.S. flocculation brings it more into line with a toxin-antitoxin flocculation, which gives the same toxin-antitoxin equivalent whether the concentration of the antitoxin or the toxin is varied. It supports the view that the α flocculating zone shows the position of neutral floccules, and gives a ready method for determining unknown concentrations of the antibody or antigen by a precipitation reaction.

Reactions between Human Sera and Sera from Rabbits Immunized to Human Sera.

In order to show possible causes of the difference between the α and β optimal zones obtained on mixing some antigens and antibodies and their identity on mixing others, the study of other precipitin reactions was undertaken. Two rabbit antihuman sera were kindly supplied by Dr. Parish, and their α and β flocculating values were determined. These were found to differ in the same way as those for antipneumococcus sera to S.S.S., as may be seen in Table IV. When the γ method was applied the optimal

TABLE IV.—*Showing the Ratios between the α and β Flocculating Zones for Whole Human Serum and Human Antiserum, and for the Water-insoluble and Soluble Fractions.*

Antigen.	Antibody.	Antigen = 1 ml. antibody. α.	Antigen = 1 ml. antibody. β.	Ratio $\frac{\alpha}{\beta}$.
Whole human antiserum	C. 77	0·093 ml.	0·0050 ml.	31·0
Human insoluble	C. 77 insoluble	0·200 ,,	<0·0050 ,,	>40·0
Human soluble	,, ,,	0·100 ,,	<0·0025 ,,	>40·0
,, ,,	C. 77 soluble	1·000 ,,	0·3100 ,,	3·2
,, ,,	,, ,,	0·100 ,,	0·0200 ,,	5·0
Human insoluble	,, ,,	0·500 ,,	0·0500 ,,	10·0
,, ,,	,, ,,	0·100 ,,	0·0200 ,,	5·0

In cases where the β flocculating value is given as $<$ a value, insufficient material was obtainable to determine the actual value.

zone was found to be more difficult to read than for the antipneumococcus sera. The floccules dispersed very readily and the more stable ones could be seen only with a lens. Shaking the tubes gently in a single rack after leaving the floccules in the water-bath overnight was found to give more easily readable zones. The results are shown in Table V; the correspondence in the values obtained by this method and by the α method is less close than with antipneumococcus flocculations.

TABLE V.—*Showing the Volume of Human Serum Equivalent to Two Rabbits Antihuman Sera as Determined with Constant Human Serum Dilutions (γ Method), and Constant Antisera Dilutions (α Method).*

Antisera.	Human serum = 1 ml. antiserum.	
	α.	*γ*.
C.77	0·093 ml.	0·12 ml.
Blend	0·810 ,,	0·66 ,,

It is obvious that such a multiple antigen as human serum will produce more than one antibody in the antisera. This statement has recently been corroborated, and the presence of both specific and non-specific optimal zones of flocculation has been shown by Goldsworthy and Rudd (1935) and Taylor and Adair (1935). Both the human serum and the antihuman serum were dialysed against distilled water, and the water-insoluble "euglobulin" separated from the water-soluble protein. The "euglobulin" fractions were made up to the original serum volume and 1 p.c. salt was added. The water-soluble fractions were made up to twice the original serum volume, and 1 p.c. salt was added. The "euglobulin" and soluble fractions of human serum were used as antigens in the flocculation reactions and the "euglobulin" and water-soluble fractions of human antiserum as antibodies. The water-soluble antibody gave two optimal flocculating zones with both antigens, which were far enough apart to be studied separately. For every fraction the α and β values were found, and where two zones occurred both were read, and the ratio between them determined. The results are given in Table IV. The nearest approach to unity ratio or to a normal diphtheria toxin-antitoxin type of reaction were the precipitin reactions of the soluble antibodies with soluble antigens. The soluble antibodies with the "euglobulin" antigen gave rather higher ratios, and the precipitin reactions given by the insoluble antibody show a ratio greater than 40. The sera from which these fractions were prepared were not absolutely specific for human serum proteins, but contained in addition some precipitins for the serum proteins of other animals. This should not affect the type of flocculation reaction. Large ratios similar to those obtained with the insoluble antibodies had been given by antipneumococcus sera (see Table I). The low potency and one other serum (see A.14577 and A.14984) gave lower ratios, which in the former case may have been due to the relatively small proportion of insoluble pneumococcus antibody present. It would seem that there is some connection between water-insoluble antibodies and a high ratio of α to β values.

It was found impossible to read the α values by means of the γ technique as all the floccules were completely dispersed.

Reactions between Dog Serum and Serum from a Horse which had been Immunized to Dog Proteins.

A small quantity of precipitin serum for dog protein was kindly supplied by Mr. Dalling. This had been made by immunizing horses to dog distemper with infected dog tissues. The precipitin serum was titrated against normal dog serum and two α flocculating zones were observed (1 and 2, Table VI).

TABLE VI.—*Showing the α and β Flocculating Zones between Normal Dog Serum, Whole and Fractionated, and Dog Autiserum, Whole and Fractionated.*

No. of reaction.	Antigen.	Antibody.	Antigen equivalent to 1 ml. of antibody. α.	β.	$\frac{\alpha}{\beta}$
1	Normal dog, Y.59	Whole serum, A.16202	0·0090	0·0086	1·04
2	,, ,,	,, ,,	0·0880	<0·0500	>1·75
3	,, ,,	Soluble, ,,	0·0079	0·0079	1·00
4	,, ,,	Insoluble, ,,	0·0260	<0·0200	>1·30
5	Soluble ,,	Soluble, ,,	0·0080	0·0071	1·12
6	,, ,,	Insoluble, ,,	0·7600	<0·0500	>15·00
7	Insoluble, ,,	Soluble, ,,	0·0900	0·0760	1·18

In reaction 1 the α and β optimal flocculating tubes coincided, and in reaction 2 there was some divergence between them. The weakness of the antiserum made the volumes to be dealt with rather large, 5–10 ml. in each tube, but, wherever it was possible, the α and β mixtures were put up in the same total volume so as to keep the conditions of observation uniform from tube to tube.

The precipitin serum was further investigated by dialysis against distilled water, and the separation of the water-insoluble "euglobulin" from the water-soluble proteins. The fractions were titrated against normal dog serum. Flocculation corresponding to reaction 1 was found in the water-soluble fraction (reaction 3 in Table VI). That corresponding to reaction 2 was found to be split up and quantitatively untraceable. Volume difficulties with the "euglobulin" fraction prevented the determination of the exact ratios between α and β values of reaction 4.

The dog serum was then dialysed, and the four reactions of the antibody and antigen treated as the fractions from the human serum and human antiserum. The reactions obtained are shown as 5, 6 and 7. From these results it would also appear that it is the water-insolubility of the antibody which is associated with the large ratios, thus confirming the work with the human antisera.

THE EFFECT OF SALT CONCENTRATION, HYDROGEN ION CONCENTRATION, TEMPERATURE VARIATION AND ANTIBODY CONCENTRATION ON CERTAIN PRECIPITIN OPTIMAL ZONES.

In the previous part of the paper all the precipitin reactions were studied in similar physical conditions. The antibody dilutions were made in 1 p.c. saline, and the antigen dilution in isotonic borate buffer at pH 8·05. The temperature used was always 40° C. The study of the variation of these physical conditions as particularly related to the reaction between pneumococcus S.S.S. and antibody was thought to be of interest, and was therefore undertaken.

Salt Concentration.

It was shown by Felton and Bailey (1926) that pneumococcus S.S.S. and antibody floccules are slightly soluble in 3 p.c. saline. This was, therefore, dissolved in 1 p.c., 2 p.c. and 3 p.c. saline respectively, and the sera were

diluted with isotonic borate buffer. Two sera were used, so that the ratio between their flocculation values as well as their actual S.S.S. equivalents could be determined. Both α and γ flocculation values were obtained and the results are given in Table VII. It will be seen that the values of the antibody equivalent vary considerably, but that the ratio between the two sera remains constant. The more salt in the flocculating mixtures, the more

TABLE VII.—*Showing Variation in the S.S.S. Equivalent to Antibody with Salt Concentration.*

Serum No.	P.c. salt.	S.S.S. = 1 ml. serum : α.	Ratio between sera.	S.S.S. = 1 ml. serum : γ.	Ratio between sera.
A.14984	1	$1{\cdot}20 \times 10^{-4}$	1 : 2·54	$1{\cdot}37 \times 10^{-4}$	1 : 2·6
A.16391		$3{\cdot}25 \times 10^{-4}$		$3{\cdot}57 \times 10^{-4}$	
A.14984	2	$1{\cdot}17 \times 10^{-4}$	1 : 2·40	$1{\cdot}03 \times 10^{-4}$	1 : 2·7
A.16391		$2{\cdot}81 \times 10^{-4}$		$2{\cdot}80 \times 10^{-4}$	
A.14984	3	$0{\cdot}72 \times 10^{-4}$	1 : 2·70	$0{\cdot}61 \times 10^{-4}$	1 : 2·9
A.16391		$1{\cdot}95 \times 10^{-4}$		$1{\cdot}77 \times 10^{-4}$	

antibody is necessary to form a stable compound with the S.S.S. It is therefore obvious that in comparing the optimal flocculating values of sera it is important to work at fixed salt concentrations. In this respect the flocculations differ from toxin-antitoxin flocculations, which give the same equivalent values unless the salt concentration is varied very widely. It would appear that the S.S.S.-antibody complex is much less stable than the toxin-antitoxin complex, and that as more antibody is required for the optimal mixture when larger quantities of salt are present, the neutral value is not obtained. This effect is very similar to that which has been shown by Bier (1931) and confirmed by Duncan (1934) of the increase in salt concentration on optimal agglutination. It was also noticed that the time for optimal flocculation was increased with increasing salt concentration, showing that 1 p.c. salt is the most convenient for practical purposes.

Hydrogen Ion Concentration.

Hydrogen ion influence on the precipitin values obtained by the titration in series of pneumococcus S.S.S. and antibody was noted by Sobotka and Friedlander (1928), and therefore some effect was to be expected when α optimal flocculating values were obtained at various hydrogen ion concentrations. In order to obtain results which could be compared with the usual values as determined when the sera are diluted in 1 p.c. saline and the S.S.S. is diluted in borate buffer, it was found necessary to dissolve the S.S.S. in B.D.H. universal buffer at the required hydrogen ion concentration to which 0·5 p.c. of salt had been added. The acidity of the mixtures was determined after flocculation had taken place, and these are the values given. This buffer saline at pH 8 was found to give values about 10 p.c. lower than those obtained with borate buffer and saline. A series of hydrogen ion concentrations was investigated and the results are shown in Table VIII. They are very similar to those for increasing salt concentration. The greater the pH, the more

TABLE VIII.—*Showing the Effect of Hydrogen Ion Concentration on the S.S.S. Equivalent to Antibody.*

pH of flocculation.	S.S.S. = 1 ml. A.14984.	S.S.S. = 1 ml. A.16391.	Ratio between sera.
8·00	$1·17 \times 10^{-4}$	$2·90 \times 10^{-4}$	1 : 2·5
6·73	$1·17 \times 10^{-4}$	$2·56 \times 10^{-4}$	1 : 2·2
6·25	$1·12 \times 10^{-4}$	$2·62 \times 10^{-4}$	1 : 2·3
6·00	$0·92 \times 10^{-4}$	$1·91 \times 10^{-4}$	1 : 2·6
5·66	$0·92 \times 10^{-4}$	$1·91 \times 10^{-4}$	1 : 2·1
4·77	$0·58 \times 10^{-4}$	$1·35 \times 10^{-4}$	1 : 2·3

S.S.S. is necessary to combine with the antibody. The appreciable effect both on the value and speed of flocculation was noted below pH 6·26. In the titration of diphtheria toxin by antitoxin no variation in the value obtained was noticed between pH 8·4 and 6·4 by Bayne-Jones (1928). With more acid solutions his results were irregular. Schmidt (1930) found that the reaction is greatly slowed at a pH value of less than 5·0, but the neutral point is obtained. In 1926, Watson and Langstaff, working with diphtheria toxoid and antitoxin, showed that the pH of the mixtures had no effect on the flocculation value of the toxoid until the precipitation level was reached. The time for optimal flocculation was increased at high pH values.

The Effect of Temperature Variation.

The effect of large temperature differences on the combination of pneumococcus S.S.S. and antibody was noted at an early stage in the work. Series of mixtures were put into the cold room overnight in the hope of delaying flocculation, and in every case the a optimal flocculating zone was found to disappear, and flocculations were found to take place in the shortest time with the highest concentration of S.S.S. In no case was an optimal zone of flocculation found when the temperature was 0–4° C., whether the range showing an optimal flocculation at 40° C. was covered or a range including much higher concentration of S.S.S. Some of the results given with two sera are shown in Table IX. The floccules formed at 0° C. are extremely stable, and do not disperse when transferred to room temperature.

The possibility of great lack of convection inhibiting the optimal zone in the cold room was considered. Corresponding flocculation mixtures were

TABLE IX.—*Showing the Effect of Large Temperature Variations on the S.S.S. and Pneumococcus Antibody Flocculation.*

Temperature.	Serum.	Volume and dilution of serum.	1 ml. of S.S.S. dilutions. 1/20,000.	1/50,000.	1/100,000.	1/200,000.	1/400,000.
0–4° C.	A.14984	1 c.c. 1/10	1	2			
40° C.	A.14984	1 c.c. 1/10			2	1	3
0–4° C.	A.14761	1 c.c. 1/20	1	2			
40° C.	A.14761	1 c.c. 1/20			2	1	3

Order of floculation = 1, 2, 3.

placed respectively in an incubator at 38–39° C., and in a water-bath at 40° C. The mixtures in the incubator took considerably longer before definite floccules circulated, but an optimal zone was obtained with each series in the same place.

The floccules formed at 0–4° C. must be an antibody-antigen complex, as neither the antibody nor the antigen precipitate at this temperature and dilution. When the antigen and antibody were mixed in the cold and then incubated as usual at 40° C. no change in the position of the optimal zone was detected.

The effect of temperature on the precipitation of Type III antibody from undiluted solutions with varying amounts of S.S.S. has recently been studied by Heidelberger and Kendall (1935). They have found more antibody nitrogen precipitated at 0° C. than at 37° C. From these results and from the reaction of the antibody with methylated S.S.S. they conclude that there is more than one antibody to Type III S.S.S.

The effect of placing human sera and antisera flocculating mixtures and dog serum and antiserum mixtures at 0–4° C. was also investigated. These were found to retain the optimal zones shown at 40° C., but combination was much slower, as with toxin and antitoxin flocculating mixtures at low temperatures.

The Effect of the Concentration of Antibody on the Velocity of the Reaction between Type I Pneumococcus S.S.S. and Antiserum A.16591.

It was suggested by Mr. Pope that more information regarding the combination of pneumococcus S.S.S. and antibody might be obtained by determining the time curve of flocculation for a series of mixtures of S.S.S. and antibody. Dilutions of S.S.S. from 1 in 5000 to 1 in 1,000,000 were made so that 10 p.c. differences in concentration were obtained in a volume of 1 ml. Five series of mixtures of serum and S.S.S. were then made. They contained respectively 1 ml. of the above dilutions of S.S.S., each with 1 ml. of serum diluted 1 in 10 in saline (series I), the further serum dilutions being, Series II, 1 in 20 ; Series III, 1 in 30 ; Series IV, 1 in 40 ; Series V, 1 in 45. Each series of mixtures was placed in a water-bath at 40° C., and the time of particulation of the mixture in each tube noted. The logarithm of the ratio of the dilution of the S.S.S. to the dilution of serum used was plotted against the time of particulation ; the curves obtained are shown in Fig. 1. From dilutions of serum of 1 in 20 to 1 in 45 it may be seen that the optimal flocculations show a very gradually decreasing ratio of S.S.S. to antibody. Further, from the shape of the curves it may be deduced that for these dilutions the S.S.S.-antibody complex is more soluble in excess S.S.S. at 40° C. than in excess antibody. With strong solutions of antibody (series I) the curve is shifted to show more rapid particulation with excess S.S.S. This resembles the shift of the optimal point at low temperatures. It is this shift which allows of the presence of the β optimum. With the serum used for these curves the β optimum at dilutions of S.S.S. 1 in 150,000 and 1 in 300,000 occurs with serum diluted between 1 in 5 and 1 in 10. It is therefore not until the antibody is undiluted, or is diluted 1 in 2, that the shift of the curve is great enough to cause an

overlapping. The reason for this shift and the presence of a β optimum is at present unknown. The idea of several antigens in S.S.S. recently expounded by Heidelberger and Kendall might tempt one to give this as the explanation for the greater affinity of S.S.S. for antibody in strong solutions. With most sera, however, as shown previously, the optimum does not appear to vary regularly with the dilution of serum and S.S.S., or it resembles a non-specific flocculation.

Comparisons between the effects of salt concentration, hydrogen ion concentration, and finally of the dilutions of antibody on the reaction between pneumococcus S.S.S. and antibody were interesting. It was noticed that

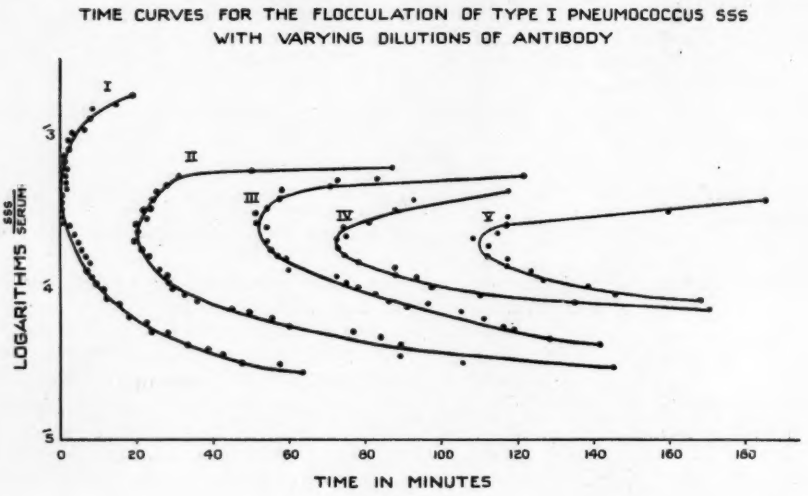

FIG. 1.

at pH 6·0 and less and a salt concentration of 2 and 3 p.c. more antibody was necessary to react with the same amount of S.S.S. in the optimal mixture. More antibody was also necessary when the reaction occurred at high antibody dilutions than at low dilutions, as shown by the velocity curves. Neutral floccules could not have been obtained in every case. The antibody-antigen complex for this reaction seems to be easily affected by physical conditions. When low dilutions of antibody were used (Curve 1) the rapidly flocculating mixtures spread into those containing excess of antigen first, as if the molecular size of the antibody were having an effect on the rate of combination. When higher dilutions of antibody were used (Curves 2–5), the optimal zone appeared in mixtures containing proportions of antigen and antibody more nearly equal to one another. The consistent influence of water-insoluble antibodies on the α and β value ratio, which was shown in the first part of the paper, further supports the idea that it is the physical properties of the antibody which most influence the reaction between the antigen and the antibody, and of these

properties the molecular size would seem to be most important. Insoluble antigens do not appear to have any such marked effect.

DISCUSSION.

The experimental work in this paper shows the existence of at least three types of precipitin reactions. First, those in which the α and β flocculating zones coincide, for example, the diphtheria toxin-antitoxin reactions; second, the type in which the α and β flocculating zones are divergent, but can be brought together by special technique, the pneumococcus S.S.S.-antibody reactions; and third, the type in which the α and β zones are always divergent, the reactions between the fractions of human serum and antiserum. Some of the probable causes of these types are the individual affinity of the antibodies for the antigens, the nature of the antigen-antibody complex and its relative solubility in excess of the antigen or antibody, and the physical properties and molecular size of antigen and antibody. The first cause is outside natural control, as antibodies produced in every animal may differ in this respect. The second and third of these causes can be controlled to a certain extent by using constant physical conditions for comparison.

Therefore, provided that physical conditions are controlled, the α and β flocculating zones will be expected to coincide if the antigen and antibody are water-soluble, and if the antigen-antibody complex is equally soluble in excess of either antibody or antigen. The zones will be expected to be divergent if the antibody is water-insoluble, and if the antibody-antigen complex is not equally soluble in antibody and antigen. The division of this class into those in which the two zones can be brought together, and those in which they are always divergent, is apparently due to the nature of the antibody-antigen complex.

It is possible that if those mixtures which were shaken up and showed no stable floccules had been allowed to flocculate again, the α zone would always have appeared first. When Duncan allowed reagglutination to take place, he found only one zone, that corresponding to our β zone. The work in this paper was completed before the publication of Duncan's work, and it has not yet been found possible to investigate this point.

SUMMARY.

1. The divergent α and β flocculating zones of pneumococcus S.S.S. and antibody have been brought together by using a special technique.

2. The necessity for controlling physical conditions when investigating precipitin reactions has been shown.

3. The partial cause of large divergence of the α and β flocculating zones in precipitin reactions has been attributed to the water-insolubility of the antibodies concerned.

I wish to thank Mr. C. G. Pope for his interest and encouragement during this work, and for helpful criticism of the manuscript, also to thank Dr. J. W. Trevan and Dr. R. A. Q. O'Meara for helpful constructive advice in writing the paper.

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INFLUENZA: OBSERVATIONS ON THE RECOVERY OF VIRUS FROM MAN AND ON THE ANTIBODY CONTENT OF HUMAN SERA.

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Received for publication November 1st, 1935.

RECOVERY OF VIRUS FROM PATIENTS.

WE have reported previously (Smith, Andrewes and Laidlaw, 1933) on the recovery of a virus pathogenic for ferrets from cases of influenza in man, and upon the failure to recover this virus from control cases. Since the publication of this paper we have attempted to infect ferrets, and in some instances also mice, with material from 53 more human subjects. The majority of these were suspected of suffering from influenza. The material, usually in the form of nose or throat washings, was supplied to us by a number of physicians, to whom we are much indebted. But inasmuch as the sources of material were very diverse, the clinical data concerning the patients concerned have not been gathered in a uniform manner, and are in many instances

meagre. Consequently we cannot at this stage attempt any correlation of the clinical and laboratory findings, though we regard this as a most important matter for future research. The data presented in the first part of this paper clearly do not take us far in deciding the difficult problem, " What is influenza ? ", but they are a record of our early attempts to recover influenza virus from human beings, and seem to us to reveal some features of interest.

Methods of Testing Material from Patients.

Patients whose secretions we wished to test were asked to gargle about 30 c.c. of saline or a mixture of saline and broth in equal parts and to spit out the garglings into a container. Sometimes nasal washings were added to the garglings. As a rule we received the material within a few hours of its being obtained and inoculated a ferret intranasally with about 1 c.c. of unfiltered material ; no anæsthetic was used for the ferrets except in a few of the later tests. The ferrets had their temperatures taken twice daily and were observed for symptoms ; if no symptoms or fever occurred, they were subsequently tested for immunity to our WS strain of virus. If we had any reason to suspect a " take " in a ferret, it was sacrificed about the fourth day after infection and passage made to another ferret. When tests were made on mice, these were inoculated intranasally under ether anæsthesia with 0·05 c.c. of material, usually unfiltered (*cf.* Andrewes, Laidlaw and Smith, 1934). They were killed and autopsied after about 5 days, whether symptoms developed or not.

Material used for inoculation was examined bacteriologically, but not in great detail. The usual practice was to sow blood-agar and Fildes' agar plates with the washings provided or with direct swabs from the patient's throat. We paid particular attention to the numbers of colonies of hæmolytic streptococci on the plate, hoping thus to separate off certain patients with streptococcal sore throats. We also noted the presence of pneumococci and *H. influenzæ*, but in no instance were these detected in very large numbers. Some of the washings were also sown on blood-agar plates and incubated anaerobically in the hope of isolating *B. pneumosintes* (Olitsky and Gates, 1921). This was done with washings from cases 29 to 36 inclusive (Table III), but no organism resembling *B. pneumosintes* was recovered. Two of these patients, as we shall record presently, yielded influenza virus.

Material from Patients not Suffering from Influenza.

Apart from the coryza cases, most of the patients shown in Table I were tested because they were originally suspected of having influenza ; they were subsequently shown to have some other adequate cause for their fever and symptoms. As will be seen from the table, virus was recovered from none of them either in ferrets or in mice. In our earlier paper we reported failure to recover virus from another case of coryza and from four normal men ; we have thus tested 18 cases which may be regarded as controls.

TABLE I.—*Tests on Material from Patients Not Suffering from Influenza.*

Patient.	Date.	Diagnosis.	Day after onset.	Recovery of virus in— Ferrets.	Mice.
1	18.iii.33	Measles	1	0	—
2	7.vi.33	Acute bronchitis	<1	0	—
3	8.vii.33	Streptococcal sore throat	1	0	—
4	27.ix.33	Coryza	1	0	—
5	18.xi.33	,,	1	0	—
6	29.xi.33	,,	1	0	—
7	9.x.34	,,	2	0	0
8	19.x.34	,,	1	—	0
9	19.x.34	,,	<1	—	0
10	10.xi.34	,,	1	0	0
11	18.xii.34	Acute sinusitis and laryngitis	1	0	0
12	30.i.35	Apyrexial sore throat	1	0	0
13	14.iii.35	Alveolar abscess	2	0	0

0 = failure to recover virus; — = no test.

TABLE II.—*Tests on Material from Patients Diagnosed as Sporadic Influenza.*

Patient.	Date.	Clinical features.	Day after onset.	Recovery of virus in— Ferrets.	Mice.
14	31.v.33	Afebrile, headache, backache, sore throat	<1	0	—
15	9.ix.33	T. 100°, headache, sore throat, pains in limbs	1	0	—
16	1.xii.33	"Typical 'flu"	3	0	—
17	12.xii.33	T. 101·5°, sore throat, malaise, backache, pains in limbs (some hæmolytic streptococci in throat culture)	1	0	—
18	3.i.34	Sore throat, headache, pains in limbs	1	0	—
19	9.i.34	T. 103·3°, pain in limbs; sore throat; later tracheitis	1	0	—
20	22.xi.34	Cold leading on to fever and tonsillitis	1	0	0
21	17.xii.34	Vomiting (suspected "gastric 'flu")	<1	0	0
22	8.i.35	Mr. Layton's case (see text)	?	0	0
23	22.i.35	Ditto	1	0	—
24	22.i.35	,,	2	0	—
25	6.ii.35	,,	?	—	0

Material from Patients Diagnosed as Influenza in Non-Epidemic Periods.

The patients in Table II were tentatively labelled influenza by their doctors, but all occurred as sporadic cases at a time of no wide prevalence and without any evidence of a high degree of infectivity.

Garglings from the last four cases in Table II (Nos. 22 to 25) were kindly sent to us by Mr. T. B. Layton, Aural Surgeon to Guy's Hospital. He has maintained (Layton, 1933) that patients with influenza show characteristic signs in the nose, tongue and fauces, which he has described as an " influenzal triad ". It will be seen that our few results afford no support for the view that the clinical picture he describes is that of the virus disease under consideration. Table II shows, in fact, that we did not recover virus from any of our twelve " sporadic influenza " cases. Some of these had symptoms closely resembling those of the epidemic influenza patients to be described next ; but in none of those observed by us was there any spread to other members of the same household.

Material from Patients Diagnosed as Influenza at Times of Epidemic Prevalence.

In the winter of 1933–34 there was very little influenza prevalent in London. There was, however, as we have previously recorded (Andrewes, Laidlaw and Smith, 1934), a small localized epidemic at the end of March, 1933. During this epidemic all six members of one household were attacked by symptoms of influenza, and from one of these (No. 26, Table III) we obtained virus. Each of two ferrets inoculated with washings from this patient showed typical fever and symptoms, and from one of them virus was carried on in series.

In 1934–35 we were fortunate in obtaining the help of Major-General Marrian Perry and other officers of the Royal Army Medical Corps, through whom we obtained throat washings from a number of cases.

During January and February, 1935, 203 cases diagnosed as influenza occurred amongst the units of the garrison at Woolwich ; this represents an incidence of about 0·5 p.c. per week. The infection was mild in character ; commonly the onset was gradual, accompanied by coryza, and the patient did not report sick until two or three days after the onset. There was little evidence of high infectivity ; the cases were diffusely scattered both as regards time and as regards the units affected. In a unit of 133 boys 16 cases occurred in one week—an incidence of 12 p.c.—but otherwise the maximum weekly incidence in any one unit was 2·4 p.c. We obtained garglings from 6 patients, in some of whom the infection was of rather more than average severity, but from none of them did we obtain virus (Cases 29 to 34, Table III).

It is arguable that the cases at Woolwich were not suffering from epidemic influenza ; in favour of this idea is the fact that the garrison was attacked in March, 1935, by a respiratory infection of a more acute nature. We were unable to test garglings from this outbreak when they were offered to us, owing to shortage of ferrets.

We have been given permission to quote from a report on the Woolwich

TABLE III.—*Tests on Material from Patients Diagnosed as Epidemic Influenza.*

Patient.	Date.	Clinical features.	Day after onset.	Recovery of virus in— Ferrets.	Mice.
26 (BW)	28.iii.34	T. 101–102°, sore throat, backache; all six members of household attacked	1	+	−
27	31.i.35	Headache, coryza, shivering, but no fever	1	0	0
28	31.i.35	"Symptoms of influenza"; four cases in family	2	0	0
29 (Woolwich)	18.ii.35	T. 100·5°, sore throat, pains in limbs	1	0	0
30 (,,)	18.ii.35	T. 99·5°, headache, sore throat, coryza	1	0	0
31 (,,)	20.ii.35	T. 102°, cough, headache, backache, sore throat, coryza	1	0	0
32 (,,)	20.ii.35	T. 99·4°, headache, malaise, coryza	1	0	0
33 (,,)	20.ii.35	T. 100°, sudden onset, headache, backache, pains in limbs, sore throat, coryza	1	0	0
34 (,,),	20.ii.35	Afebrile, headache, abdominal pain	1	0	0
35 (Shorncliffe) (SC)	7.iii.35	Severe case; 3 days' pyrexia, pharyngitis, headache, backache	3	+	0
36 (,,) (BA)	7.iii.35	T. 101·6°, sore throat, backache, headache	3	+	0
37 (,,) (ST)	7.iii.35	T. 102°, 4 days' pyrexia, headache, sore throat, coryza	3	+	−
38 (,,)	7.iii.35	"Severe uncomplicated case"	4	0	−
39 (,,) (PE)	7.iii.35	T. 101·6°, 3 days. pyrexia, coryza, pharyngitis	4	+	−
40 (,,)	7.iii.35	"Severe uncomplicated case"	4	0	−
41	13.iii.35	Afebrile, pains, headache, nausea, W.B.Cs. 3800 per c.mm.	1	0	0
42	13.iii.35	72 hours' pyrexia, headache, malaise, pains in limbs; W.B.Cs. 8000 per c.mm.	1	0	0
43 (CA)	15.iii.35	3 days' fever (100°), coryza, headache, sore throat, malaise; hæmolytic streptococci predominant in throat washings	1	+	0
44	16.iii.35	2 days' fever; relapse later; headache, pyrexia, tonsillitis; W.B.Cs. 8200 per c.mm.	1	0	0
45 (Shorncliffe) (CH)	18.iii.35	T. 101·8°; "uncomplicated influenza"	1	+	−
46 (,,) (DO)	18.iii.35	T. 101·2°; "uncomplicated influenza"	1	+	−
47	23.iii.35	Pyrexia 48 hours, headache, pains in limbs, coryza; W.B.Cs. 9000 per c.mm.	?	0	−
48 (BH)	25.iii.35	Fever and malaise. Ambulatory for 7 days; then fatal pneumonia and empyema (Group IV pneumococcus)	1 or 2	+	−

Patients from whom virus was recovered are designated by letters as well as numbers; these have been used to identify the virus strains.

influenza outbreak by Major S. Smith of the Royal Herbert Hospital at Woolwich :

"There were two main 'waves', the first during January and the first three weeks of February, and the second during the whole of March ; separated by a more or less quiescent period during the last week in February.

"The second wave started early in March with almost explosive onset ; during the week ending March 6th, 98 cases were admitted, 500 cases being admitted during the month.

"The admissions for influenza during the first five months have been :

"208	cases during	January.
216	,,	February.
500	,,	March.
122	,,	April.
21	,,	May (to date).

"During February :

"71	cases were admitted	during	the	week	ending 6.ii.35.
66	,,	,,	,,	,,	13.ii.35.
42	,,	,,	,,	,,	20.ii.35.
28	,,	,,	,,	,,	27.ii.35.
98	,,	,,	,,	,,	6.iii.35.

"It is a notable fact (as far as this hospital is concerned) that when the daily admissions fell below a certain level the cases became less typical, and many anomalous cases, presenting no very definite symptoms (more or less conforming to severe common cold), were admitted to the influenza wards.

"During this quiescent period the onset is apt to be gradual, often extending over several days ; the temperature only a few points above the normal ; slight headache, rapid resolution and convalescence, etc. Whereas, as occurred in March, when the daily admission-rate was high, cases conformed much more closely to the classical picture of epidemic influenza : sudden onset of symptoms, the patient usually giving a history of sudden onset, either on the day of admission, or the day previous ; severe catarrhal symptoms ; chest pain ; a high proportion showing definite basal congestion, which might or might not progress to pneumonia ; high continued fever ; severe headache, which might arouse a suspicion of meningitis ; prolonged convalescence, etc.

"It was during a relatively quiescent period, when anomalous cases were occurring, that most of the washings were taken for ferret inoculation (*i. e.* February 18th–21st). It is suggested that if further washings had been taken during March when the second wave was at its height, a proportion of positive results would, in all probability, have resulted.

"Thirty-six cases progressed to pneumonia (3·2 p.c.) ; a high proportion of these—7 (19·4 p.c.)—progressed to empyema."

In February and March, 1935, an epidemic of upper respiratory infection attacked troops at Dover and Shorncliffe. The onset was in general more sudden than in the Woolwich cases tested by us ; further, the infectivity was higher, at least in certain units. Thus, in one battalion 82 men, 14·5 p.c. of the strength, were admitted to hospital in one week in mid-February. In the first two weeks of March 45 cases occurred in a regiment of Hussars ; the incidence per week was 5·3 and 4·7 p.c. respectively. Two other units were involved in the third week of March, with an attack-rate of 6 p.c. for that week. In mid-March numerous cases occurred in a military boys' school at Dover. A hundred cases occurred in one week—an incidence of 25 p.c. ; in the following week there were 30 cases (incidence, 7·3 p.c.). We obtained garglings from a number of cases from Shorncliffe and tested the most promising on ferrets and mice. Eight lots of garglings were tested in all, and virus was recovered in every instance except two. We may have obtained the garglings

too late in these two cases, as they were each tested on the fourth day of illness. Cases 35, 36, 37, 40, Table III, were from the Hussars mentioned above, and, as will be seen, three out of four yielded us virus. Two lots of garglings were tested from the outbreak in the school (Cases 45, 46), and virus was obtained from each.

In addition to the army cases, we tested in the early months of 1935 material from eight patients in London. Virus was obtained from two only. One of them, No. 48, Table III, provided us with washings early in his disease, but thereafter, although probably febrile, he continued at work. On the seventh day after the onset he developed pneumonia, and subsequently died from a complicating pneumococcal empyema.

Attempted Recovery of Virus from Post-Mortem Material.

We tested in ferrets or in ferrets and mice, emulsions of lungs obtained *post mortem* from five patients believed to be suffering from influenzal pneumonia, but we recovered no virus. In every instance the lungs had the gross appearance characteristic of this disease; they were described as being deep red in colour, with, in some instances, frothy hæmorrhagic fluid exuding on section, and with areas of hæmorrhagic broncho-pneumonic consolidation varying in extent in different cases (see Table IV).

TABLE IV.—*Tests on Post-mortem Material ("Influenzal Lungs").*

Patient.	Date.	Clinical and post-mortem features.	Day after onset.	Recovery of virus in—	
				Ferrets.	Mice.
49	19.iv.33	T. 105°; child of 11 months	2	0	—
50	14.iii.35	T. 103·4°; exacerbation of chronic bronchitis	5	0	0
51	25.iv.35	Æt. 17; early hæmorrhagic bronchopneumonia; W.B.Cs. 37,500 per c.mm.	1	0	0
52	12.v.35	Ante-mortem thrombi and staphylococci in lungs	about 9	0	0
53	26.vi.35	Æt. 18; sudden death	?<1	0	0

In two of the patients (Nos. 50 and 52, Table IV) the illness apparently began as an exacerbation of chronic bronchitis. In one of them (52) ante-mortem thrombi were found histologically in some of the pulmonary blood-vessels, and clumps of cocci could be detected in these thrombi. Staphylococci also grew out in almost pure culture from the lungs. Others of the lungs examined gave a very rich bacterial growth, and cannot be regarded as ideal material for the isolation of virus. Lungs from the last two cases in Table IV (52 and 53) were obtained at times when influenza was not locally prevalent.

ADAPTATION OF HUMAN VIRUSES TO MICE.

When we reported the susceptibility of mice to influenza in July, 1934, we hoped that a readily accessible method of detecting influenza virus in

human throat washings had been discovered. But Tables I to IV show that in no instance did we recover a virus by inoculating garglings directly into mice. Material from three human patients which proved infective for ferrets failed to produce lesions in mice (Table III, patients 35, 36, 43). It was thought that minimal quantities of virus might be present in these mouse lungs ; accordingly in two of these three instances the lungs of the mice were pooled, ground up and used to infect more mice ; yet these, too, appeared normal when the mice were killed 6 days later.

Only after a number of passages in ferrets did we succeed in infecting mice with the virus (see Table V). As will be seen, the BH strain proved much more difficult than the others to adapt. In almost every instance the adaptation appeared to be gradual ; only one, two or three of six mice would be infected at the first successful attempt, the proportion of positives rising in the course of two or three passages.

TABLE V.—*Adaption of Virus to Mice.*

Strain.	Attempts to infect mice. Unsuccessful.	Successful.
BA	Direct from man	After 3 ferret passages.
SC	After 1 ferret passage	,, 3 ,, ,,
PE	,, 1 ,, ,,	(No further tests.)
CA	Direct from man	After 2 ferret passages.
CH	..	,, 2 ,, ,,
DO	..	,, 2 ,, ,,
BH	After 3, 5, 7 and 9 ferret passages.	,, 13 ,, ,,

SEROLOGICAL IDENTIFICATION OF NEW VIRUS STRAINS.

We have already recorded that the BW strain isolated in March, 1934, appeared to be identical with our WS (1933) strain. In the endeavour to

TABLE VI.—*Cross-immunity Tests between Virus Strains in Ferrets.*

Number of ferret.	Strain with which previously infected.	Strain with which test was made.	Result of test.	Result of test in control ferret.
F. 7	WS	SC	—	+
F. 70	SC	WS	—	+
F. 8	WS	BA	—	+
F. 72	BA	WS	—	+
F. 44	WS	BH	—	+
F. 96	BH	WS	—	+
F. 82	WS	CA	—	+
F. 90	CA	WS	—	+
F. 87	WS	CH	—	+
F. 89	WS	DO	—	+
F. 8	WS and BH	Porto Rico 8	—	+
F. 44	WS and BH	Philadelphia	—	+

+ Indicates fever and nasal symptoms. — Indicates absence of fever and nasal symptoms.

discover whether the same was true of the 1935 viruses we first carried out cross-immunity tests in ferrets; the results of these are shown in Table VI. These findings clearly indicate a close serological relationship between the old and new strains, but do not permit us to conclude that they are identical. For the human (WS) and swine influenza viruses are shown by serological tests in mice to be immunologically distinct; yet they have some antigenic relationship and protect, one against the other, in a proportion of ferrets (Smith, Andrewes and Laidlaw, 1935). Accordingly, after adaptation of the virus to mice, we determined whether filtrates of the new strains were neutralized by the immune horse-serum which we had previously studied (Laidlaw, Smith, Andrewes and Dunkin, 1935). We felt that if this had good neutralizing power against all the filtrates, there were unlikely to be any great serological differences amongst them. The technique of the neutralization test will be described later in this paper. We found that all the new strains were neutralized by our IH2 horse-serum in approximately the same dilution as was the homologous WS strain. A protocol of one experiment is given in Table VII.

TABLE VII.—*Neutralization of Eight Influenza Strains by IH2 Serum.*

	Control mice inoculated with virus saline mixture.	Mice inoculated with mixtures of virus and IH2 serum. Final dilutions of serum in mixture.		
		1 in 20.	1 in 100.	1 in 500.
SC virus	+++	000	000	000
BA ,,	+++	000	+00	+00
CA ,,	+++	000	000	+00
CH ,,	0++	000	+00	+++
DO ,,	+++	+00	000	+++
Philadelphia virus	+++	000	000	000
Porto Rico 8 virus	++0	000	000	+00
WS virus	+++	000	000	+00

Each sign represents a mouse. + = typical influenza lung lesions found at autopsy. 0 = lungs normal.

Dr. T. Francis, at the Hospital of the Rockefeller Institute, New York City, has described the recovery of a number of strains of human influenza virus in America. He kindly sent us two of these, labelled "Porto Rico 8" and "Philadelphia", in the form of dried mouse lungs. We readily infected ferrets and mice with this material, and found that both strains cross-immunized with our WS virus in ferrets and were neutralized in mice by our IH2 serum to full titre (Table VII). Francis (1935) has already recorded similar findings as regards cross-immunity between the English and American strains.

ANTIBODIES IN HUMAN SERA.

Experiments in Ferrets.

Our earlier attempts to detect in human sera neutralizing antibodies against the WS strain of virus were carried out in ferrets. A centrifuged suspension of nasal mucosa from an infected ferret was mixed with an equal

volume of the serum to be tested, and 1 c.c. of the mixture, after standing on the bench for 10 minutes, was instilled into the nostrils of the test ferret. The animal was then observed for the development of fever and nasal symptoms. Some human sera, tested by this technique, completely inactivated the virus, but the results were irregular, and in later tests virus suspensions were diluted 1 in 100 before testing them against human sera. Eighteen sera were so tested. Nine were from patients recently convalescent from influenza and all of these neutralized the 1 in 100 virus, though one failed to do so when the same sample was retested later. Of nine sera from people who had not recently suffered from influenza, seven had demonstrable antibodies. Quantitative data could not readily be obtained in work with ferrets, and our later experiments were therefore carried out in mice.

Technique of Mouse Tests.

The use of mice in neutralization tests has been described in an earlier paper (Laidlaw, Smith, Andrewes and Dunkin, 1935). In brief, undiluted membrane filtrates of infected mouse lungs were mixed with equal volumes of the serum to be tested, or with dilutions thereof, and the mixtures inoculated intra-nasally into anæsthetized mice. As a rule the sera were tested in falling fivefold dilutions. The mice were killed, usually on the fifth day after infection, and their lungs examined for lesions. The way in which results were read is illustrated in Table VIII. A few samples of human sera were apparently toxic for mouse lungs, leading to the production of greyish lesions in the lungs when inoculated in a 1 in 2 dilution ; 1 in 10 dilutions of these sera were always innocuous.

Two points regarding technique require special mention at this stage.

First, as our routine method, we held virus-serum mixtures in contact for only 10 minutes at room temperature before testing them. In order to make sure that this procedure was justifiable, we carried out the following experiment : Virus filtrate and dilutions of IH2 serum were held in contact for 2 hours at 37° C. and inoculated into mice ; *post mortems* on the mice revealed in due course that neutralization had occurred when the serum was in a final dilution of 1 in 100, but not in one of 1 in 500. When virus and serum-dilutions were held separately for 2 hours at 37° C. and mixed only a few minutes before testing, exactly the same end-point was obtained ; the 2 hours' incubation had not affected the result. This conclusion was confirmed in another experiment, in which, again, the serum dilution which would inactivate strong virus filtrate was determined. When, however, we observed the effect of incubation on the titration of sera against dilute virus filtrates (final dilution 1 in 1000 or 1 in 2000) irregular results were obtained. It is possible that with more dilute reagents, incubation may affect the outcome.

The second point in technique concerns the reference of the human sera to a standard. Our titrations were carried out during a period of twelve months, and against virus filtrates of necessarily varying potency. In order that these titrations might be comparable, we tested in every experiment the immune horse-serum, IH2, described in an earlier paper (Laidlaw, Smith, Andrewes and Dunkin, 1935). The human sera were assessed in terms of

this serum ; a few were better than standard (IH2), some about equal to standard, others equal to one-fifth, one twenty-fifth of standard and so on. Whatever the variations in the potency of filtrates, IH2 serum hardly ever failed in a 1 in 20 final dilution to neutralize undiluted virus filtrate, and never succeeded in inactivating in a 1 in 1250 or 2500 dilution ; the strength of virus did, however, affect the results with the 1 in 100 and 1 in 500 serum-dilutions. In tests against swine influenza virus we used as standard the horse-serum IH4, the preparation of which we have described (Laidlaw, Smith, Andrewes and Dunkin, 1935) ; this was approximately as active against swine influenza virus as was serum IH2 against the WS strain.

TABLE VIII.—*Protocol of a Neutralization Experiment Against WS Virus.*

Serum dilution.	IH2 (standard).	A æt. 8.	B æt. 6.	C æt. 11.	D æt. 13.	Controls (broth-saline instead of serum).
1 in 2	..	..	222	000	200	..
1 in 10	000	000	333	233	330	..
1 in 50	200	230	..	..	..	444444332
1 in 250	233	333	..	..	..	..

Each figure in this table represents a mouse. 0 = mouse with no detectable lung lesions post-mortem ; 1 = mouse with lesions less than 1 mm. in diameter ; 2 = mouse with lesions 1–3 mm. in diameter ; 3 = mouse with fairly extensive lung lesions ; 4 = mouse with almost complete hepatization of lungs, nearly always a dead mouse.

An illustrative protocol will make the scheme clearer (Table VIII). From the results of this experiment we gave the four children's sera tested the following approximate values : A = standard (S), B < S/25, C = S/5 or S/25, D = S/25. Serum A was tested in higher serum-dilutions than the others because a preliminary experiment had shown that it was a potent one. Many sera were tested thus in two stages, a second test being carried out if the serum-dilutions used in the first experiment proved unsuitable. In all, 30 sera were titrated on more than one occasion, and in every instance consistent results were obtained.

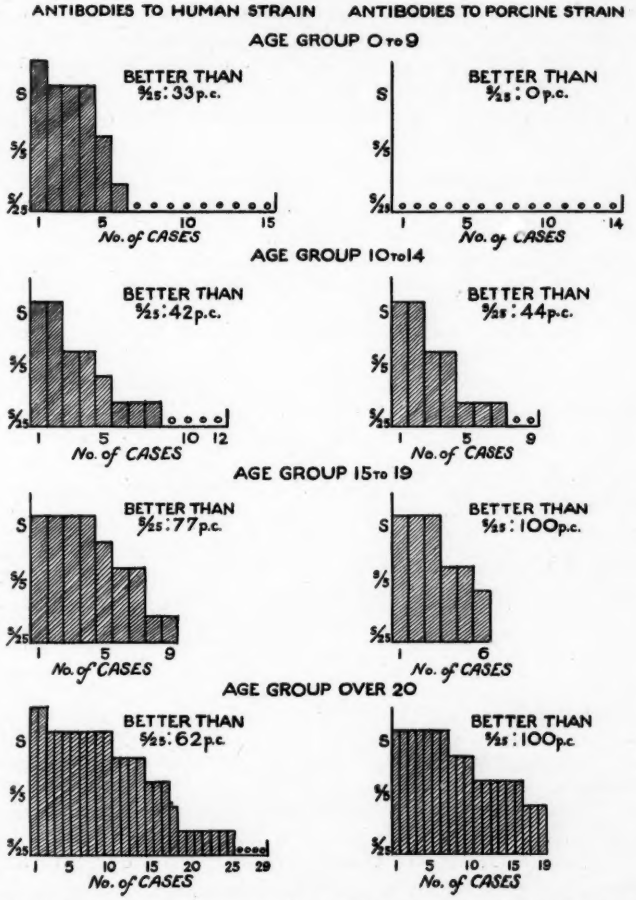
We realized that since we were using groups of only three mice for each dilution our estimations of the strength of a serum were only approximate.

Antibodies to WS Virus in Human Sera.

On the left-hand side of the chart is shown the occurrence of antibodies to WS virus in persons of different age-groups. As the chart shows, some neutralizing activity could be detected in nearly all adult human sera, but very differing amounts were present in different sera. No significant differences were found amongst the different age-groups of adults, and accordingly all adults have been placed in one group in the chart. As is indicated in the chart, the results for the adult sera have been drawn to half the scale, so that the eye may more readily compare the incidence of antibodies in the different groups. It is so difficult to obtain a reliable history as to past influenza in an individual that we did not attempt to obtain histories from most of the people furnishing serum ; one man, however, who had very good antibodies,

was confident that he had never suffered from " flu ". We examined two sera taken from soldiers at Shorncliffe (Cases 35 and 36, Table III) 12 days after

Anti-influenzal Antibodies in Human Sera of Various Age-Groups.


ANTIBODIES TO HUMAN STRAIN
ANTIBODIES TO PORCINE STRAIN
AGE GROUP 0 TO 9
BETTER THAN S/25 : 33 p.c.
BETTER THAN S/25 : 0 p.c.
AGE GROUP 10 TO 14
BETTER THAN S/25 : 42 p.c.
BETTER THAN S/25 : 44 p.c.
AGE GROUP 15 TO 19
BETTER THAN S/25 : 77 p.c.
BETTER THAN S/25 : 100 p.c.
AGE GROUP OVER 20
BETTER THAN S/25 : 62 p.c.
BETTER THAN S/25 : 100 p.c.
No. of CASES

Each vertical column represents a serum ; the height of the shading indicates the antibody titre. Sera are graded as better than S (standard IH2 or IH4 horse-serum), equal to S, S/5, or S/25, with intermediate grades for sera between S and S/5 or between S/5 and S/25. Spaces marked 0 indicate sera with no antibody or with less than S/25.

the onset of an attack of influenza. We had actually recovered virus from these two men ; both of them had very good antibodies in their " convalescent " sera, equal to standard or better. One patient was bled soon after an attack

of influenza in February, 1933, and again in December, 1934, nearly two years later. The recently convalescent serum was about equal to our standard, the later sample probably about one-fifth as good.

Dr. Stuart Mudd, of the University of Pennsylvania School of Medicine, Philadelphia, kindly sent us some sera from patients taken 5 to 10 weeks after an attack of influenza and also some pooled control sera. Dr. T. Francis isolated a virus from this outbreak in Philadelphia, and this proved serologically identical with our WS strain (Francis, 1935). The sera had been dried after freezing *in vacuo* and were sent to us dried in lyophile form (*cf.* Mudd, Reichel, Flosdorf and Eagle, 1934). These sera had, on the whole, rather poor neutralizing power, estimated as follows :

C 91 = S/25 . C 111 > S
C 107 = S/25 . C 121 = S/25
C 109 = S/5

Three of the lots of pooled control sera from Philadelphia had titres respectively of S, S, and S/25 ; as they were pooled sera, they have not been included in the chart. Later Dr. Mudd sent us some more convalescent influenza sera obtained 1 to 6 weeks after an attack. These had been collected by Drs. Pettit and Pepper from Eskimos in Alaska at the end of an epidemic which occurred there in April and May, 1935. The potency of these was estimated as shown below :

E. 1, æt. 22	.	S to S/5	.	E. 10, æt. 17	. S
E. 2, ,, 60	.	S	.	E. 11, ,, 21	. S/25
E. 3, ,, 40	.	S/25	.	E. 12, ,, 15	. S
E. 4, ,, 16	.	S	.	E. 13, ,, 42	. S/5
E. 5, old	.	S/25	.	E. 14, ,, 18	. >S/25
E. 6, æt. 63	.	<S/25	.	E. 15, ,, 19	. S/5
E. 7, old	.	S	.	E. 16, ,, 25	. <S/25
E. 8, æt. 44	.	S/5 to S/25	.	E. 17, ,, 19	. S

These results are included in the chart.

It will be noticed that all but two, Nos. 6 and 16, had demonstrable antibodies in their sera, but that there was no evidence in the group as a whole of higher antibody titres than we had met with in adults with no recent history of influenza. No relationship was observed between severity of attack, as recorded by Drs. Pettit and Pepper, and titre of serum ; nor did the time of bleeding, whether 1, 2, 3 or 6 weeks after the attack, have any obvious significance.

The Alaskan sera were, for the most part, sent to us dried in the lyophile form ; the method of drying preserves both antibodies and complement. In order to exclude the possibility that the drying had damaged their anti-influenzal properties, we tested two sera which had been sent in liquid form side by side with reconstituted, dried samples of the same sera : with neither serum was any difference between the two samples found.

The chart shows that children under 10 have significantly lower antibodies in their sera than have older children and adults. Sera equal to the standard

are less frequent, and more than half have no demonstrable neutralizing power. Four sera, coming from children aged 4, 6, 6 and 8, stand out in contrast to the others as being very potent. Of these, the child of 8 was said by her parents to have had influenza in 1933, but no history of any illness suggesting influenza was obtained from the parents of the other three children. Most of the children's sera we examined were kindly obtained for us by Dr. C. H. Stuart-Harris.

Antibodies to Swine Influenza Virus in Human Sera.

The occurrence of antibodies against the porcine strain of virus is illustrated in the right-hand half of the chart. We have shown previously (Smith, Andrewes and Laidlaw, 1935) that human and porcine strains of influenza are immunologically distinct, but yet exhibit some overlap when cross-neutralization tests are made with potent sera. Such an overlap does not account for the occurrence of antibodies to swine influenza in human sera, for a number of sera with good antibodies to the porcine strain were almost without effect on the human, and *vice versa*.

The chart shows that in the sera from adults and from children over 10, antibodies to swine influenza are present as regularly as are those active against the human virus. In fact, amongst adult sera we found no examples with antibodies very low or absent. All the more striking is the fact that 14 sera from children under 10 contained no demonstrable antibody at all. The ages of these children were as follows, the numbers of sera examined in each age-group being given in brackets: 1 (1), 2 (1), 3 (1), 4 (1), 5 (3), 6 (3), 7 (1), 8 (1), 9 (2). A sudden change is evident when we pass to the groups aged 10 or over; three children of 10 all had good antibodies in their sera (S/5 or better), and three aged 11 also showed neutralizing power (equal to standard in one serum and to S/25 in the other two). We shall consider the significance of these findings shortly.

Comparison with American Workers' Findings.

Drs. Francis, Magill and Shope in the United States have been carrying out studies on the antibody-content of human sera at the same time as ours. They have kindly allowed us to refer to their results, which will be published shortly (Francis and Magill, 1935 or 1936; Shope, 1935 or 1936). Their technique has differed somewhat from ours, since they have tested the sera against unfiltered 10 p.c. suspensions of mouse lungs instead of against filtrates. Further, they have evaluated their results in terms of the extent of lesions developing in mice receiving the mixtures of virus and undiluted serum. Most of the sera from Philadelphia and from Alaska which we studied have been examined by them also, so that it has been possible to compare the two techniques directly. Their results accord very closely with ours, and confirm the very striking fact, brought out in our studies, that there is a great contrast between the rarity of antibodies to porcine virus in children under 10, and their frequency in those over that age.

ATTEMPT TO INFECT HUMAN VOLUNTEERS.

We naturally desired to know whether virus which had been repeatedly passed through ferrets would produce influenza in man. A number of volunteers, mostly students from St. Bartholomew's Hospital, offered to allow themselves to be inoculated for the purpose of this test, and Prof. F. R. Fraser undertook to observe them clinically. In order to minimize the risk of untoward bacterial complications, we examined their naso-pharyngeal bacterial flora on three separate days within a week before the test was made. It was decided to reject volunteers who showed hæmolytic streptococci or pneumococci in their throat cultures or *H. influenzæ* in more than very small numbers. Throat swabs were plated out on blood-agar and Fildes' agar, and attempts were also made to detect potentially pathogenic organisms by intraperitoneal and subcutaneous inoculation into mice of broth cultures from swabs. Sera of volunteers were also examined for neutralizing antibodies to WS virus. At first a number of volunteers were rejected because their sera neutralized the virus when tested on ferrets. But we later found that almost all human sera contained antibodies, and we were unable to obtain volunteers whose sera had none. Accordingly we tested two students with virus, although antibodies were present in their sera. Each was rigidly isolated in a one-bed ward for 48 hours before the test, and then 2 c.c. of filtrate of turbinate suspension from an infected ferret was instilled, 1 c.c. into each nostril. One man, inoculated in September, 1933, received virus which had been repeatedly passed through ferrets in the course of 6 months. The other received on March 26th, 1934, virus which had only undergone 5 ferret passages, had then been stored in the dried state for 9 months and finally passed through 2 more ferrets before being used for the test.

Neither volunteer showed any abnormal signs or symptoms whatever after inoculation. The result of this experiment, though disappointing, did not lead us to conclude that the virus under study was not the causative agent of influenza; there are several possible explanations for the failure to infect the volunteers. First, it is obvious that the men may have been immune; this immunity may or may not have been directly connected with the neutralizing antibodies in their sera. Secondly, ferret-passage may have attenuated the virus for man. Thirdly, the virus may have to be accompanied by a *Hæmophilus* or other organism to produce disease in man; in that case influenza of man would resemble swine influenza, for the causation of which both virus and a bacillus (*H. influenzæ suis*) are necessary (Shope, 1931).

DISCUSSION.

We have recovered a virus pathogenic for ferrets from 14 cases of influenza, 5 in 1933, 1 in 1934 and 8 in 1935. All our successes occurred at times of prevalence of a form of upper respiratory infection of high infectivity. From sporadic cases diagnosed as influenza no virus was recovered. These findings support the view, which is perhaps self-evident, that what is commonly regarded as influenza is not an entity. It becomes, therefore, a matter of prime importance to find, if possible, some means of differentiating clinically

between the disease caused by a ferret-pathogenic virus, which we identify as epidemic influenza, and other conditions resembling it. It is, of course, possible that some of the sporadic cases are due to an influenza virus in which lessened infectivity for man is associated with failure to adapt itself to the ferret.

The finding of antibodies active against the human strain of virus in most adult sera is of great interest, and needs further study in relation to immunity or susceptibility to infection. At present we are quite ignorant as to whether people having antibodies in their sera are immune to an attack of the disease or not. Francis and Magill (1935) found in persons in the acute stage of influenza no antibodies to the virus, but these developed during convalescence. We have recorded (Smith, Andrewes and Laidlaw, 1935) that in ferrets the immunity wanes during the six months following infection, and that the animals have regained some susceptibility at a time when antibodies are still demonstrable in their sera.

The antibodies to swine influenza in adult human sera may possibly be non-specific in the sense that they represent past contact, not with that virus, but with some unknown related antigen. But if we regard them as possibly specific, an interesting field for speculation and research is opened up. Swine influenza is prevalent in the Middle West of the United States, and is said not to have occurred amongst the herds of pigs there until the time of the 1918 pandemic. Koen (1922–23) believed that the disease of pigs then prevalent was identical with that of man. In support of this possibility is the fact, recorded by Elkeles (1935), that the human strain of influenza virus will infect pigs. It is unlikely that the Londoners whose sera we examined acquired any infection from pigs, especially since swine influenza has not yet been recognized in England. We have therefore to face the possibility that human beings in England and America have been infected by, or exposed to an influenza virus serologically similar to or identical with the swine influenza virus ; and there is the further possibility, already discussed by one of us (Laidlaw, 1935), that this virus is that responsible for the 1918–19 pandemic. The age-distribution of antibodies to the porcine virus is now seen to be of some interest ; the absence of neutralizing sera in children under ten may be due to complete or partial dying out of this particular strain of virus as a cause of human disease during the last decade. It is true that antibodies to many viruses and other antigens are low in young children ; but the data in this paper show in the lowest age-group a contrast between antibodies to human and to swine influenza viruses which is probably significant, especially in view of its confirmation from the United States. Further evidence that no virus closely related serologically to swine influenza is now widely prevalent as a human infection, is afforded by the fact that all the strains hitherto isolated by us in England or by Francis in America have proved to be antigenically like our WS strain.

SUMMARY.

1. A virus pathogenic for ferrets was recovered from 8 cases of epidemic influenza in the early months of 1935.

2. Six of the new strains were studied, and found to be serologically identical with the WS virus encountered in 1933.

3. No success was met with in attempting to infect mice directly with human throat washings. Five of the new virus strains, however, infected mice after 2 or 3 passages through ferrets ; one (BH strain) required 13 ferret passages before it would do so.

4. No virus was recovered from cases of common cold or sporadic influenza.

5. Neutralizing antibodies to human (WS) influenza virus were found in the majority of human sera examined. Their incidence was rather lower in children under ten than in older children and adults.

6. Neutralizing antibodies to swine influenza virus were regularly found present in adult sera but were wholly absent from the sera of fourteen children under ten.

7. Influenza virus passaged through ferrets failed to infect two human volunteers by the intranasal route. Both these volunteers had neutralizing antibodies in their sera before the test was made.

We wish to express our thanks to those who have kindly furnished us with clinical material, and in particular to Major-General Marrian Perry, Major L. M. Rowlette, Major R. N. Phease, and Major S. Smith of the R.A.M.C., Prof. F. R. Fraser, Dr. G. H. Faulkner, Mr. T. B. Layton, Dr. Stuart Mudd and Dr. C. H. Stuart-Harris.

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GRADED COLLODION MEMBRANES FOR BACTERIOLOGICAL STUDIES. PRACTICAL ASPECTS OF THE MECHANISM DETERMINING THE CHARACTER OF THE MEMBRANE, AND THE ROLES OF PARTICULAR SOLVENT CONSTITUENTS.

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Received for publication November 5th, 1935.

STUDIES on the formation and properties of collodion films (Elford, 1930) demonstrated two important facts ; (1) that uniformity of membrane structure could best be ensured by allowing the system to gel spontaneously and preferably in a gradual manner, and (2) that the porosity of the film was determined largely by the state of aggregation of the nitrocellulose particles at the instant when gelation commenced. A new series of graded collodion membranes was prepared (Elford, 1931) by a method in which the essential feature was the incorporation in the collodion of components which permitted a controlled variation of the aggregation factor. The mutual antagonism in their solvent properties for nitrocellulose shown by acetone and amyl alcohol was exploited for this purpose.

In preparing a membrane, the collodion, consisting of nitrocellulose dissolved in an appropriate mixture of ether, ethyl alcohol, acetone and amyl alcohol, is poured on a flat horizontal glass cell to form a layer 1 to 2 mm. thick. This is exposed in a still atmosphere at standard temperature and humidity, the solvent evaporating freely. Each component evoporates at a different rate, and each rate changes with time, so that the process is an extremely complex one. As the evaporation proceeds, the concentration of nitrocellulose increases, as do the proportions of the less volatile components of the solvent mixture. The ratio of amyl alcohol to acetone increases, and the mutual antagonism of these solvents induces aggregation of the nitrocellulose particles and may finally result in coagulation. Another tendency which becomes increasingly pronounced as the evaporation proceeds is that to gel formation, *i. e.* the tendency for the nitrocellulose particles to link up in the formation of a porous matrix. These two processes undoubtedly determine the structure of the final film. If the aggregation is very advanced when gelation sets in, then a highly porous membrane results.

Role of the Individual Components in the Solvent Mixture.

The contributions of the various individual components in the solvent mixture towards one or other of the competing processes—improved solution and aggregation—may be studied by use of the " water titre " as an index

of the solvent power. The " water titre " or " tolerance " is the amount of water necessary to produce in the system a certain arbitrary standard turbidity. The solvent powers and the compatibilities of various components may be compared in this way. It frequently happens that the solvent behaviour of a mixture is quite different from what one might expect from the individual properties of the constituents ; sometimes solvent power is greatly increased (*e. g.* alcohol and ether), and sometimes reduced (*e. g.* acetone and amyl alcohol). Titrations upon relatively simple systems have indicated that the relationship between solvent power and the properties of individual components becomes increasingly complex as the number of components grows and their compatibilities become involved. Generally, the hypothesis that an increase in the proportion of a good solvent favours dispersion and retards the aggregation which produces a membrane of lower porosity, while the addition of a non-solvent promotes aggregation and hence results in a more permeable membrane, has been confirmed. To present the mass of physical data accumulated will not be attempted here, as it is hoped by extended investigations along these lines to acquire yet further knowledge of the more complex conditions in the cell during membrane formation. However, not only can such studies throw light upon the processes determining the nature of the collodion films—strength and porosity—but they also have immediate practical value as a guide to the best means for correcting membrane abnormalities, particularly in regard to sediment formation.

Sediment Formation.

Elford (1931) emphasized that a good membrane must lift away from the cell easily and leave the glass surface perfectly clear and free from any deposit. One batch of commercial necol used by us yielded membranes which were definitely more porous than usual, and, in addition, with the " parent collodion," (see Elford, 1931) a sediment invariably remained in the cell after coagulation. While it is known that considerable variation may occur among different samples of nitrocellulose, the fact that, apart from this one instance, the necols have behaved with marked consistency, makes it more probable that the explanation lay in the particular necol having possessed a higher water content than normally. This being so, in the light of our earlier considerations, the obvious way to correct such sediment formation is to promote the gelling tendency and repress aggregation, so that gelation may set in before aggregation reaches the point of precipitation. This may be accomplished by adding a small amount (0·1 to 0·2 p.c. will probably suffice) of acetic acid or ethylene glycol monoethyl ether. This substance is non-volatile, therefore its effect increases as the evaporation of the other solvents proceeds, and the coagulation is held in check.

Amyl Alcohol used in Preparing Membranes.

The amyl alcohol used in preparing gradocol membranes must have been thoroughly washed free of organic impurities. The presence of such impurities may cause tiresome irregularities and make the films adhere to the glass cell.

Such were our experiences with systems made from a particular batch of amyl alcohol, which upon test was found to contain abnormally high organic impurities. When amyl alcohol is shaken with an equal volume of concentrated sulphuric acid, the colour produced should not be more than a golden yellow. The British Drug Houses A.R. product, "pyridine and nitrogen free", has conformed with the desired standard.

Use of Ethylene Glycol Monoethyl Ether in Obtaining Membranes of Low Porosities.

A series of membranes with porosities progressively less than 0·7 to 0·8μ is obtained by incorporating small amounts of acetic acid with the "parent

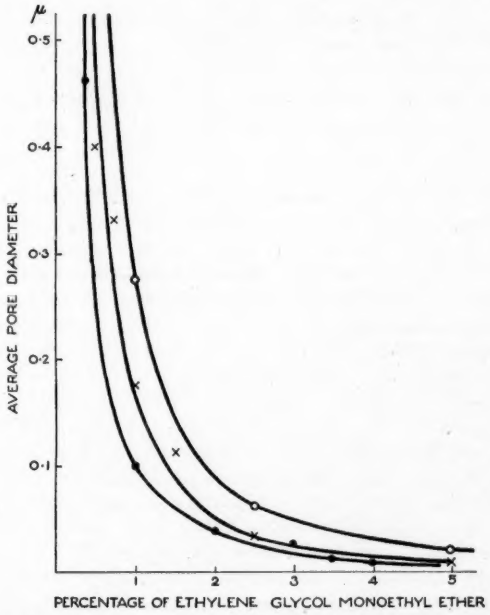

FIG. 1.—Variation of average pore-diameter (A.P.D.) with percentage of ethylene glycol monoethyl ether. The three curves refer to different series of membranes.

collodion" (Elford, 1931). An equally good series of graded membranes may be prepared by using ethylene glycol monoethyl ether instead of acetic acid. Ethylene glycol monoethyl ether is an excellent solvent for nitrocellulose, and we have used the British Drug Houses preparation after redistilling and collecting the fraction at 133·5° C. The secondary collodion systems are prepared by making additions (by weight) of the ethylene glycol monoethyl ether to the "parent collodion". Fig. 1 shows how the porosities were found to vary with increasing additions to "parent collodion" from three different

batches of necol. The films are generally somewhat stronger than those in the corresponding acetic acid series, and equally uniform. The ethylene glycol monoethyl ether is a colourless and odourless liquid, which is much more conveniently handled than glacial acetic acid.

Dryness of Solvents.

Elford (1931) emphasized the necessity for purifying and drying the various solvents used in making collodion membranes. To obtain the best reproducibility dry pure solvents are essential, but, as Bauer and Hughes (1934) have recently pointed out, very good uniform membranes may be prepared using ordinary absolute alcohol which has not been specially dried over quicklime. Such films will be slightly more permeable than those prepared from the dry alcohol, but the procedure may prove quite satisfactory so long as the degree of absoluteness of the alcohol remains constant.

Calibration of Collodion Membranes—an Amplifying Note on the Assumed Membrane Structure.

Elford and Ferry (1935) described in detail the technique for calibrating collodion membranes, and discussed the several assumptions made in calculating their average pore diameters. It was shown that the only structure which satisfactorily accounts for the high specific water content (S) values, and conforms with the mechanism of film formation, is that in which the pores are distributed in more than one direction, and also intersect one another. An arrangement of independent non-intersecting pores equally distributed in three mutually perpendicular directions, in which case the volume, V_e, of the effective pores is equivalent to S/3, was rejected on grounds of incompatibility with the observed high values of S. Dr. Manegold (Göttingen), in a personal communication, has pointed out that in the paper referred to, the data of Bjerrum and Manegold (1927) were mistakenly discussed as appertaining to a system of *independent* pores (non-intersecting) distributed uniformly in *three mutually perpendicular directions*, when they actually relate to a system of *independent* pores distributed haphazardly in *all directions*. We much regret this misunderstanding, and thank Dr. Manegold for kindly bringing it to our attention. Although the same pore radius values hold for both structures, the number of pores per sq. cm. of membrane surface differs in the two instances, being given by $S/3\pi r^2$ for the perpendicular orientation and by $S/2\pi r^2$ for the general distribution (r = pore radius). Thus the high figures for the total volume of pores per c.c. in Table II of Elford and Ferry's paper are explained and must be discounted. However, the main objection to such a structure with independent pores is in no way affected by this.

In the calibration of membranes the assumption is made that the volume of the effective pores is equivalent to the specific water content, S, a relationship which, strictly, holds good only for unidirectional capillaries at right angles to the membrane surface. Elford and Ferry estimated that, applied to a system of intersecting cylindrical pores distributed uniformly in three directions perpendicular to the faces of a cube, this assumption would result in the

calculated pore diameter being in error by a factor 1·3 to 1·4. The view was expressed that this was probably an over-estimate of the error involved in actual practice, since no account had been taken of possible orientation among the pores.* Consideration of the very ideal system of pores uniformly distributed in three mutually perpendicular directions as before, but also regularly arranged so that their axes intersect in points forming a cubic-lattice, shows the factor of error involved to be only 1·15 to 1·25. For pores of square cross-section arranged in this way, the common volume at each intersection is a cube, and the relation between V_e, the volume of effective pores perpendicular to any one face of the cube, and V_p, the total pore volume, is readily shown to be—

$$V_p = 3V_e - 2V_e \sqrt{V_e}.$$

Thus the more regular and symmetrical the system of intersecting pores, and, of course, the more closely they are packed, then the nearer does the calculated value of the average pore diameter, based on the assumption $V_e = S$, approach the true value.

SUMMARY.

1. The underlying principle of the method used in obtaining a graded series of collodion membranes ("gradocol" membranes) may be said to be the controlled variation of the aggregation of the nitrocellulose relative to the incidence of spontaneous gelation. If the aggregation is advanced in relation to the gelling tendency, then a more porous film is obtained, while the porosity is decreased when the aggregation factor is repressed.

2. The means of remedying sediment formation has been discussed in the light of the mechanism of film formation.

3. The use of ethylene glycol monoethyl ether in obtaining membranes with graded porosities has been described.

4. An amplifying note in justification of the membrane structure assumed in calibrating collodion membranes emphasizes anew that, if the pores are postulated as not all mutually parallel, they must be considered as intersecting and not independent.

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* [*Note.*—The last item in the first column of Table III in Elford and Ferry's paper was intended to read $\rightarrow 0$, thus indicating, in the line, that as S tends towards zero, the value of ϕ approaches 0·33. When $S = 0$, then ϕ ceases to have any real meaning.]

FURTHER STUDIES ON THE DIFFERENTIATION OF THE VIRUS OF VESICULAR STOMATITIS FROM THAT OF FOOT-AND-MOUTH DISEASE, WITH PARTICULAR REFERENCE TO A RAPID AND CERTAIN METHOD OF RESOLVING MIXTURES OF THE TWO VIRUSES.*

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Received for publication November 12th, 1935.

COTTON (1926) stated that " though vesicular stomatitis is not a disease that is directly of great economic importance it does, because of the similarity of its lesions in cattle to those of foot-and-mouth disease, greatly complicate the diagnosis of the latter. It is easy to mistake the one disease for the other. Any work, therefore, that will make it possible to distinguish certainly and quickly the one from the other is worth while ".

Before proceeding with the main theme of the present paper, it appears necessary to point out that considerable doubt still exists in the minds of some as to the occurrence of foot as well as mouth lesions in animals affected with vesicular stomatitis, and that Bekker, de Kock and Quinlan (1934) have recently quoted an erroneous statement on this point by Eaton (1933), viz. " Vesicular Stomatitis—here only mouth lesions develop and consist of vesicles usually seen only on the tongue, but occasionally on the gums. Important differential characteristics are the absence of feet lesions and no locomotory disturbances ". Although in early descriptions of the disease it was stated that the lesions of vesicular stomatitis were confined to the mouth and tongue, Cotton (1926, 1927) reported that foot as well as mouth lesions may occur in both cattle and horses affected with vesicular stomatitis, although perhaps with less frequency than in cattle affected with foot-and-mouth disease, and that the lesions are indistinguishable from those of the latter disease. Olitsky, Traum and Schoening (1926–7) observed also that swine infected with the virus of vesicular stomatitis may show lesions, including those on the feet, indistinguishable from the lesions of foot-and-mouth disease. Further, vesicles may also be seen as in foot-and-mouth disease on the teats of cows affected with vesicular stomatitis. It is now generally agreed that the two diseases are clinically indistinguishable in cattle, swine and guinea-pigs.

In view of controversy it is apparently also essential to stress the fact (see also Galloway and Elford, 1931) that although vesicular stomatitis has been considered generally to be a less contagious disease than foot-and-mouth disease and presumably, therefore, of less economic importance, it may sometimes (Cotton, 1927) assume the characteristics of a contagious epizootic disease.

In our previous paper (Galloway and Elford, 1933) the chief contrasts between the characteristics of the two diseases were tabulated. It was pointed out that the differentiating features were not absolute, or such as would wholly

* This work was done on behalf of the Foot-and-Mouth Disease Research Committee, who have kindly given their permission for the publication of this paper.

exclude the possibility of the virus of vesicular stomatitis being a variant of the virus of foot-and-mouth disease, to which it was believed by some to bear a generic relationship. The fact that the virus of vesicular stomatitis was shown to have a size value about eight times that estimated for the virus of foot-and-mouth disease was regarded as the most direct evidence of the distinct individuality of the two diseases. Later, when it was found possible (Burnet and Galloway, 1934) to propagate the virus of vesicular stomatitis in the chorio-allantoic membrane of the developing hen's egg, and it was established that the virus of foot-and-mouth disease, on the other hand, did not survive under similar conditions, the idea suggested itself that those two facts might be employed to form the basis of a simple and economical technique for a rapid and certain resolution of mixtures of the two viruses.

It may be recalled that the Foot-and-Mouth Disease Commission of the United States Department of Agriculture (1928) suggested the possibility of vesicular stomatitis and foot-and-mouth disease being present in the same herd of animals or indeed in the same animal simultaneously. Although the Commission made no experiments on mixtures of the two viruses, they suggested the following procedure as a means of deciding whether a mixed infection existed: "With the suspected material one or more horses and two or more cattle are inoculated by scarification of the mucous membrane of the gum or dental pad of cattle. It is of course essential that susceptible animals be used. The development of the disease in the horse and the cattle would indicate that the inoculated material contains at least the virus of vesicular stomatitis. Two or more normal cattle are now inoculated with the material collected from the horse. This should contain only vesicular stomatitis virus assuming that the foot-and-mouth disease virus (if present in the original material) had been lost because of the unsusceptibility of the horse to infection with foot-and-mouth disease. Thus we have two groups of cattle immune to subsequent exposure to the virus with which they were inoculated: Group A inoculated with the original material and Group B inoculated with that derived from the horse. After an interval of one week, both groups are injected with the original material, which should be kept in a cold chamber in phosphate buffered 50 p.c. glycerol. Then:

"1. If animals in Group A are unaffected and those in Group B are infected, it would indicate that the original material contains both vesicular stomatitis and foot-and-mouth disease.

"2. If animals in Groups A and B are unaffected, it would indicate that the original material contained only the virus of vesicular stomatitis."

Quite apart from the cost and time necessary for such an experiment, the results that would be obtained might quite conceivably be other than those expected. It is assumed for one thing that the inoculation of a supposed mixture into the horse would eliminate the virus of foot-and-mouth disease, but there is no assurance that this would be so. More experimental evidence is necessary as regards the susceptibility of the horse to inoculation with different strains of the virus of foot-and-mouth disease. Although there are no accurate experimental observations on record which suggest that horses contract foot-and-mouth disease under natural conditions, the evidence available is insufficient to determine whether or not some sort of a lesion may not be produced by inoculation of this virus directly into the mucous membrane of the mouth. Rabbits, for instance, do not appear to become infected with foot-and-mouth disease under natural conditions, but they develop very definite vesicular lesions when the virus is inoculated intralingually and the virus can be passaged from rabbit to rabbit in series.

While the suggestion that animals might be simultaneously affected with the two viruses under natural conditions must be borne in mind, the possibility must also be considered that under experimental conditions one virus might become accidentally contaminated with the other. Whatever the occasion, the usefulness of an efficient and easy way of determining the presence of each virus and of separating the two is apparent.

Before proceeding with the main experiments to determine the practicability of the suggestion that a combination of filtration and inoculation of eggs might be employed to form the basis of a technique for the resolution of mixtures of the two viruses, it was found to be necessary to re-examine the filtering properties of the virus of vesicular stomatitis in the light of certain observations made in the experiments in which it was propagated in developing hen's eggs. The conclusions drawn from the result of these further observations on filtration are fully discussed in the first section of this paper.

It was also imperative to carry out a more extended series of experiments on the results of the inoculation of the virus of foot-and-mouth disease into eggs. These experiments are described in the second section of this paper.

In the third section the series of investigations which were made on the resolution of mixtures of the two viruses are described in detail, and include those experiments made to determine whether the two viruses can be passaged together in guinea-pigs.

The fourth and last section deals with experiments made to confirm and extend the observations of Pyl and Klenk (1933), Pyl (1934), and Reppin and Pyl (1934), who have advanced yet another method of distinguishing between the virus of vesicular stomatitis and that of foot-and-mouth disease.

I. FILTRATION EXPERIMENTS ON THE VIRUS OF VESICULAR STOMATITIS.

In a previous paper, Galloway and Elford (1933), the results of filtration experiments were recorded which showed that the virus of vesicular stomatitis has a diameter of about 70–100 mμ., and that this size value, which is the same for both the " Indiana " and " New Jersey " strains, is about eight times that previously estimated for the virus of foot-and-mouth disease (8–12mμ.). Later Burnet and Galloway (1934) reported that it is possible to propagate the virus of vesicular stomatitis in the chorio-allantoic membrane of the developing hen's egg, and the most important fact, that the inoculation of eggs renders possible the detection of small amounts of the virus of vesicular stomatitis which may produce no reaction by direct inoculation into guinea-pigs. This fact suggested that a re-examination should be made of the results of the filtration experiments previously reported on. A number of further filtration experiments have been made with both strains of virus, and the filtrates have been tested on guinea-pigs and also inoculated into eggs. The results are recorded in Table I (the Indiana strain) and Table II (the New Jersey strain).

The first experiment was made with virus (Indiana strain) that had received several alternate passages through guinea-pigs and eggs (Table I, V.S. $E._x$ $G.P._x$ (1)). A filtrate through a membrane of A.P.D. 0·13μ produced lesions in guinea-pigs as well as in eggs. This result was contrary to previous experience with virus which had been passaged only through guinea-pigs (Galloway and Elford, 1933), for this consistently gave inactive filtrates with membranes of an A.P.D. 0·13μ. Suspensions of the chorio-allantoic membrane of eggs inoculated with the filtrates were injected into guinea-pigs, and the experiment was repeated (V.S. $E._{x+1}$ $G.P._{x+1}$ (2)) with a similar result. Although a filtrate employing a membrane of A.P.D. 0·13μ produced lesions in guinea-pigs and eggs, a filtrate employing a membrane of A.P.D. 84 mμ. produced

TABLE I.—*Filtration of the Indiana Strain of Vesicular Stomatitis Virus Before and After Passage in Eggs.*

History of virus.	Titre of stock filtrate in—		Tests on filtrates.									
			A.P.D. 0·17μ.		A.P.D. 0·14μ.		A.P.D. 0·13μ.		A.P.D. 0·12μ.		AP.D. 84 mμ.	
	Eggs.	Guinea-pigs.	Eggs.	Guinea-pigs.	Eggs.	Guinea-pigs.	Eggs.	Guinea-pigs.	Eggs.	Guinea-pigs.	Eggs.	Guinea-pigs.
V.S. N.T.E. [G.P.] (1)	1 : 100,000	1 : 1000±	++	++	−	−	00	00	−	−	−	−
V.S. N.T.E. [G.P.] (2)	1 : 100,000	1 : 10,000	++	++	−	−	00	00	−	−	−	−
V.S. N.T.E. [G.P.] (3)	1 : 100,000	1 : 1000±	−	−	+0	00	−	−	−	−	−	−
V.S. N.T.E. [G.P.] (4)	1 : 100,000	1 : 10,000	−	−	+0	00	−	−	00	00	−	−
V.S. N.T.E. [G.P.] + egg membrane (5)	1 : 100,000	1 : 10,000	++	++	−	−	00	00	−	−	−	−
V.S. N.T.E. [G.P.] (6)	1 : 100,000	1 : 1000	−	−	Undiluted ++ 1 : 100 00	Undiluted 00 1 : 100 00	−	−	00	00	−	−
*V.S. $E._X$ $G.P._X$ [G.P.] (1)	1 : 100,000	1 : 1000	++	++	−	−	++	++	−	−	−	−
*V.S. $E._{X+1}$ $G.P._{X+1}$ [G.P.] (2)	1 : 100,000	1 : 1000	++	++	−	−	++	+0	−	−	00	00
V.S. $E._1$ [E.]	1 : 10,000	<1 : 100	−	−	−	−	++	00	−	−	00	00
V.S. $E._1$ $G.P._1$ [G.P.] (1)	1 : 100,000	1 : 10,000±	++	++	−	−	00	00	−	−	−	−
V.S. $E._1$ $G.P._1$ [G.P.] (2)	1 : 100,000	1 : 10,000	++	++	−	−	++	00	−	−	−	−
V.S. $E._2$ $G.P._1$ [G.P.]	1 : 100,000	1 : 10,000±	−	−	−	−	++	00	00	00	−	−
V.S. $E._3$ $G.P._1$ [G.P.]	1 : 100,000	1 : 10,000	−	−	−	−	++	00	−	−	−	−
V.S. $E._2$ $G.P._2$ [G.P.]	1 : 100,000	1 : 1000	−	−	−	−	++	00	−	−	−	−
V.S. $E._5$ $G.P._1$ [G.P.]	1 : 100,000	1 : 1000	−	−	−	−	−	−	00	00	−	−
V.S. $E._{11}$ $G.P._1$ [G.P.]	1 : 100,000	1 : 1000	−	−	++	++	−	−	00	00	−	−
V.S. $E._{16}$ $G.P._1$ [G.P.]	1 : 100,000	1 : 1000	−	−	Undiluted ++ 1 : 100 ++	Undiluted +0 1 : 100 00	−	−	00	00	−	−

V.S. N.T.E. = Virus not passaged through eggs.

V.S. E. = Virus passaged through eggs.

V.S. E. G.P. = Virus passaged through eggs and guinea-pigs. The figure after E. or G.P. signifies the number of passages.

* The number of passages in eggs and guinea-pigs which had been made in these two cases was probably about 10 (1) and 11 (2). The virus had been passaged alternately in eggs and guinea-pigs.

[E.] = Source of virus was chorio-allantoic membrane of an infected egg.

[G.P.] = Source of virus was lymph from pad vesicles of infected guinea-pigs.

++ = Lesions in both of two eggs or guinea-pigs inoculated.

+0 = Lesions in one egg or guinea-pig.

00 = Lesion in neither of two eggs or guinea-pigs inoculated.

− = No test made.

N.B.—Control passages to guinea-pigs were made of chorio-allantoic membranes from inoculated eggs.

(1) and (2) etc. = Different stock filtrates were used.

TABLE II.—*Filtration of the New Jersey Strain of Vesicular Stomatitis after Passage in Eggs.*

History of virus.	Titre of stock filtrate in eggs.	Tests on filtrate A.P.D. 0·13 μ in— Eggs.	Guinea-pigs.	Remarks.
V.S. N.T.E. [G.P.]	1 : 100,000	00	00	V.S. N.T.E. = Virus not passaged through eggs.
V.S. $E._1$ [E.]	1 : 100,000	+0	00	V.S. E. = Virus passaged through egg.
				V.S. E. G.P. = Virus passaged through egg and guinea-pig.
V.S. $E._1$ $G.P._1$ [G.P.]	1 : 100,000	++	00	The figure after E. or G.P. signifies the number of passages.
V.S. $E._2$ $G.P._1$ [G.P.]	1 : 100,000	++	00	[E.] = Source of virus—the chorio-allantoic membrane of an infected egg.
V.S. $E._2$ $G.P._4$ [G.P.]	1 : 100,000	+0	00	[G.P.] = Source of virus—guinea-pig vesicle lymph.
V.S. $E._2$ $G.P._{11}$ [G.P.]	1 : 100,000	00	00	++ = Lesions in both of two eggs inoculated.
V.S. $E._2$ $G.P._{13}$ [G.P.]	< 1 : 100,000	00	00	+0 = Lesions in one of two eggs inoculated.
V.S. $E._2$ $G.P._{15}$ [G.P.]	1 : 100,000	+0	00	00 = Lesions in neither of two eggs or guinea-pigs inoculated.

no reactions either in eggs or guinea-pigs. A re-examination was then made of virus which had been stored in the dried state at—4° C. to 0° C.,* and which had not been passaged at any time through eggs (V.S. N.T.E.). Filtrates of such virus through a membrane of A.P.D. 0·13μ produced no reactions in either guinea-pigs or eggs. Further, guinea-pigs inoculated with these filtrates, when subsequently tested, did not prove to be immune. This observation confirmed our previous results.

A series of experiments was then made with virus that had been passaged one or more times through eggs (V.S. $E._1$ $G.P._1$ . . . V.S. $E._5$ $G.P._1$), etc., and the results showed that filtrates through membranes of A.P.D. 0·13μ, but not of a lesser porosity, generally contained virus in sufficient amount at any rate to infect eggs. One passage through eggs was not always sufficient to effect this change (*v.* Table I, V.S. $E._1$ $G.P._1$ (1)). All the experiments except one were made with the lymph taken directly from the pad vesicles of guinea-pigs. The exception was an experiment, V.S. $E._1$ E., made with virus contained in a suspension prepared from the chorio-allantoic membrane of an inoculated egg. Although the stock filtrate prepared from this was of a lower titre than that usually obtained with guinea-pig virus, the result obtained by secondary filtration through a membrane of A.P.D. 0·13μ was similar to that observed in the case of guinea-pig lymph from animals inoculated with egg virus. Another

* The virus of vesicular stomatitis has been found to keep well *in vacuo* or in sealed tubes at − 4° C. to 0° C. after rapid and thorough drying over P_2O_5 *in vacuo*. Two different samples in sealed tubes of dried vesicle lymph + skin from guinea-pigs infected with the Indiana strain were proved infective on the 245th and 589th day respectively. A similar sample of the same strain kept *in vacuo* was active on the 303rd day. Three different samples in sealed tubes of the New Jersey strain were still active on the 245th, 453rd and 495th days respectively. Similar material kept in 50 p.c. glycerine has proved frequently to be non-infective after 180 days.

series of experiments was made with the New Jersey strain of virus with similar results (Table II).

In order to determine whether or not these results were due to some physical effect resulting from the presence of a substance in the chorio-allantoic membrane of the egg, which might become associated with the virus and persist even after several passages through guinea-pigs, virus which had not been passaged through eggs was mixed with a suspension in broth of the ground-up chorio-allantoic membrane of an uninoculated egg (Table I, V.S. N.T.E. + egg membrane). A stock filtrate was then prepared from the mixture, and secondary filtrates obtained through membranes of A.P.D. 0·17μ and 0·13μ respectively. The filtrate through A.P.D. 0·17μ contained sufficient virus to infect both guinea-pigs and eggs, while the filtrate through A.P.D. 0·13μ was not infective either for eggs or guinea-pigs.

The possibility of extracts or filtrates of the chorio-allantoic membrane having some effect on the permeability of guinea-pig tissues to virus was also considered. Three experiments were made in the following way: Stock broth filtrates of guinea-pig (only) passaged virus were prepared. The filtrates were divided into three equal portions. A series of ten-fold dilutions in broth of each sample was made. To each dilution of the first series was added an equal amount of broth, to each dilution of the second series an equal amount of unfiltered chorio-allantoic membrane extract, and to each dilution of the third series an equal amount of a permeable membrane filtrate of this extract. Guinea-pigs were then inoculated with the dilutions from the three series. No evidence was adduced that the addition of the egg membrane extract or filtrate of extract had any consistent or noticeable effect on the results of the titrations (Table III).

TABLE III.—*Effect of Addition of Egg Membrane (Extract or Filtrate) on Titration of Vesicular Stomatitis Virus in Guinea-pigs.*

	Titre of stock filtrate alone.					Titre of stock filtrates + egg membrane extract (unfiltered).				
	1:10.	1:100.	1:1000.	1:10,000.	1:100,000.	1:10.	1:100.	1:1000.	1:10,000.	1:100,000.
(1)	++	++	++	00	00	++	++	++	+0	+0
(2)	+0	+0	00	00	00	+0	00	00	00	00
(3)	++	++	+0	+0	00	++	++	+0	+0	00

Titre of stock filtrate + filtrate of egg membrane extract.				
1:10.	1:100.	1:1000.	1:10,000.	1:100,000.
—	—	—	—	—
++	+0	00	00	00
++	++	++	+0	00

++ = Lesions in both guinea-pigs inoculated.
+0 = Lesions in one of two guinea-pigs inoculated.
00 = Lesions in neither of two guinea-pigs inoculated.

In another series of experiments virus which had been passaged through eggs was given several consecutive passages through guinea-pigs, and then re-examined to observe whether it had reverted in respect of filtering properties

to those of a guinea-pig (only) passaged virus (Table II, V.S. $E._2$ $G.P._1$, V.S. $E._2$ $G.P._4$ . . . $G.P._{11}$ . . . $G.P._{13}$, etc.). The evidence obtained suggested that a gradual reversion of the virus was taking place, but that it was incomplete, even after ten or more consecutive passages in guinea-pigs.

The filtering properties of virus passaged through guinea-pig only and virus which at some stage in its history had been passaged through eggs were further compared by the use of membranes of A.P.D. 0·14μ, 0·12μ and 84 mμ. It will be seen from Table I that membranes of A.P.D. 0·14μ allowed more of the egg-passaged virus (V.S. $E._{11}$ $G.P._1$ and V.S. $E._{10}$ $G.P._1$) to pass than of the guinea-pig (only) passaged virus (V.S. N.T.E. (3), (4) and (6)), but no virus passed through membranes of A.P.D. 0·12μ (6 experiments) or A.P.D. 84 mμ (2 experiments). This was true even of virus which had been passaged as many as 10 or 11 times consecutively in eggs.

From a general consideration of these results it appears that vesicular stomatitis virus which has been passaged in eggs filters a little more easily than the virus which has been passaged only in guinea-pigs. Out of a total of 11 experiments with virus which at some stage in its history had been passaged in eggs, active filtrates through membranes of an A.P.D. of 0·13μ were obtained in 10 instances. On 2 occasions sufficient virus passed through such membranes to infect both guinea-pigs and eggs, and in the other 8 instances to infect only eggs. In the one experiment in which an inactive filtrate was obtained the virus had been passaged only once through eggs. Out of a total of 8 experiments (Tables I and II, also Table V, Experiment 4 in Section III) with virus which had not at any time been passaged through eggs, with membranes of the same porosity, filtrates non-infective for either eggs or guinea-pigs were obtained in all 8 cases. In addition, in two experiments with egg-passaged virus and membranes of an A.P.D. of 0·14μ, more virus was detected in the filtrates than in any of three similar filtrates of guinea-pig (only) passaged virus. These results cannot be explained either by a higher titre of the virus after passage through eggs or to the presence of some substance in the chorio-allantoic membrane, aiding in some way the passage of the virus through collodion membranes (diminished surface tension), or increasing the permeability of the tissues of the inoculated guinea-pigs to virus. The only hypothesis which appears tenable is that the virus itself is slightly modified either as regards size or surface properties by repeated passage through eggs. The modification, although definite and not easily reversible, is not great, for, even after 11 consecutive passages of the virus through eggs, inactive filtrates were obtained with membranes of an A.P.D. of 0·12μ or less (6 experiments). In the filtration experiments previously reported (Galloway and Elford, 1933), 11 experiments were made with membranes of A.P.D. of 0·13μ, and in none of these was a filtrate infective for guinea-pigs obtained, and, moreover, the guinea-pigs inoculated with these filtrates did not develop any immunity. In the present series of experiments, guinea-pigs inoculated with 0·13μ filtrates which were shown to contain some virus by the egg tests were often, although not invariably, found to be immune on reinoculation with virus, although they had shown no lesions. This is additional evidence of the accuracy of the observations made on guinea-pig (only) passaged virus as compared with egg-passaged virus.

The evidence of this extended study does not in any way adversely affect our original conclusion as to the relative sizes of the viruses of vesicular stomatitis and foot-and-mouth disease. It confirms and amplifies the previous work. It is interesting to observe that Bauer and Cox (1935) have obtained a very similar filtration end-point for the virus of vesicular stomatitis to that recorded by us. They employed the same strains of virus as those examined by us, and maintained them either in chick-embryo tissue cultures or mouse brain. Their test animal was the mouse. They obtained inactive filtrates in four experiments with membranes of an A.P.D. of 0·13 μ, and active filtrates in two experiments out of four with membranes of A.P.D. 0·14μ.

II. INOCULATION OF THE VIRUS OF FOOT-AND-MOUTH DISEASE INTO DEVELOPING HEN'S EGGS.

A total of 58 developing hen's eggs have been injected in different experiments with an inoculum of about 0·05 c.c. of filtrates in broth of the virus of foot-and-mouth disease. This quantum of virus was deposited on the surface of the chorio-allantoic membrane of 9–10-day-old embryos at the base of an artificially produced air-sac.* Twelve of these eggs were opened about the 24th hour, 30 about the 48th hour and 6 on the 3rd, 4th, 5th and 6th days. In no case was the virus recovered from the chorio-allantoic membrane of the inoculated eggs and no specific lesions were observed. The embryos themselves were also unaffected and were proved to be free of virus by guinea-pig tests. The majority of these experiments were made with the G.F. strain of virus, which is of the Vallée O type, but a limited number of additional experiments have also been made with three other strains of virus, two of the Vallée O type and one of the Waldmann and Trautwein C type, with similar results.

Recently Frenkel and van Waveren (1933–34) reported that they had inoculated a total of 86 hen's eggs with filtrates in physiological saline of the virus of foot-and-mouth disease. Twenty-nine of these were inoculated with guinea-pig virus and 57 with swine virus. One of us (I. A. G.) has had an opportunity of discussing with these workers the technique employed by them. It is clear that their method of inoculating eggs differed from ours in that they did not deposit the virus on the chorio-allantoic membrane at the base of an artificially produced air-sac, as was done in our experiments. The amount of virus inoculated was greater than in our tests, and varied from 2 to 15 drops (about 0·1 c.c. to 0·8 c.c.). No evidence of multiplication of the virus was obtained, but in 10 instances virus was recovered at the 24th to 48th hour from eggs inoculated with swine virus, and in 2 instances at the 24th hour from eggs inoculated with guinea-pig virus. When 15-day-old embryos were employed, the virus survived in a larger proportion of eggs than when eggs incubated only 6–10 days were inoculated.

A further series of experiments was made, therefore, to determine whether survival of the virus in the egg might occur if the technique of injection was modified in certain respects. Some experiments were made with 15-day-old

* A fuller description of these experiments is given in the appendix of the 5th Progress Report of the Foot-and-Mouth Disease Research Committee.

embryos, and others in which the amount of virus inoculated into the egg was increased to 0·5 c.c. Ten 15-day incubated eggs were inoculated with 0·05 c.c. of guinea-pig virus filtrate in broth ; 5 of these were opened at the 24th hour and 5 at the 48th hour. No virus was recovered from any of them. Ten eggs were inoculated with 0·2 c.c. to 0·5 c.c. of virus filtrate, and these were opened at about the 24th hour. Virus was recovered from one egg inoculated with 0·5 c.c. of virus, but of two guinea-pigs inoculated intradermally into the pads with an emulsion of the membrane from the egg, one only showed lesions. A portion of the membrane from this egg was ground up in broth, and 0·05 c.c. amounts of the suspension inoculated into 2 other eggs in which no virus was detected after 24 hours' incubation.

It seems reasonable to assume that the results obtained by Frenkel and van Waveren, who recorded occasional survival of the virus of foot-and-mouth disease in eggs, can be explained by the technique employed for inoculation. Virus injected in larger amounts than 0·05 c.c. may come in contact with parts of the egg other than the chorio-allantoic membrane, in which situations it may survive. It is known that under more favourable conditions than appear to be presented by the medium of the chorio-allantoic membrane of the developing hen's egg as, *e. g.*, in buffered phosphate solution at pH 7·5, the virus may survive at 37° C. for as long as 4 to 5 days, even when not in high concentration. It is also very probable that the inoculation of a relatively large amount of foreign protein into the egg may embarrass the normal functions of the chorio-allantoic membrane.

III. RESOLUTION OF MIXTURES OF THE VIRUS OF VESICULAR STOMATITIS AND THE VIRUS OF FOOT-AND-MOUTH DISEASE.

Experiment 1 (*see also Chart I*) : *Examination of "Unknown" Virus.*

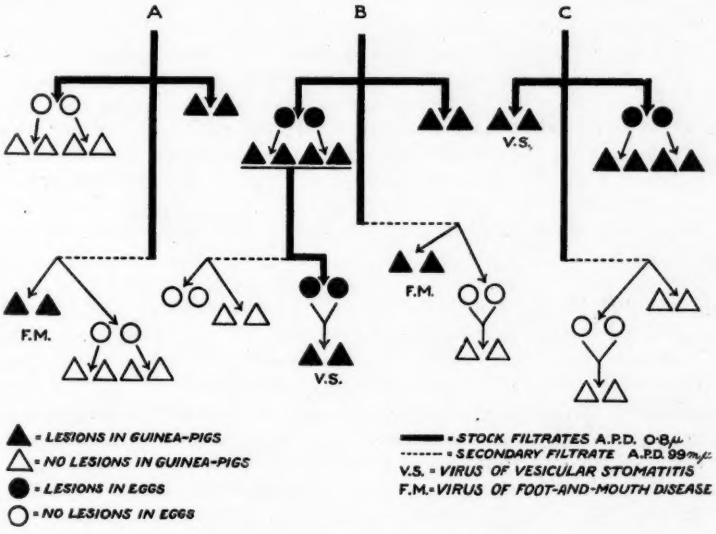
Stock broth filtrates (membranes A.P.D. 0·8μ) of the virus of vesicular stomatitis and foot-and-mouth disease were prepared, and submitted to an independent observer, who subsequently handed back to the writers three tubes labelled A, B, and C. One of these "unknowns" was stated to contain only the virus of foot-and-mouth disease, another only the virus of vesicular stomatitis, and the remaining tube a mixture of equal parts of the two viruses.

Samples from each of the three tubes were inoculated intradermally into the pads of two guinea-pigs and also into two eggs. The guinea-pigs inoculated with samples from tubes A and B developed primary lesions after 24 hours and generalized lesions after 48 hours, while those inoculated with the sample from tube C developed local lesions after 48 hours. This was suggestive, but not conclusive, evidence that tubes A and B contained the virus of foot-and-mouth disease. The eggs inoculated with the samples from tubes B and C developed lesions, and virus was recovered from them by guinea-pig inoculation. This was evidence that tubes B and C contained the virus of vesicular stomatitis. The eggs inoculated with material from tube A remained normal and no virus could be demonstrated by subinoculations into guinea-pigs. This evidence in itself strongly suggested that tube A contained only the virus of foot-and-mouth disease. Secondary filtrates through membranes of A.P.D. 99 mμ.

were prepared by filtering samples of virus from tubes A, B and C, and were each inoculated intradermally into the pads of 2 guinea-pigs and into 2 eggs. Those guinea-pigs inoculated with secondary filtrates from A and B developed generalized lesions. None of the eggs inoculated with the three secondary filtrates developed lesions, nor could any virus be recovered from them by inoculation of guinea-pigs. This confirmed the previous supposition that tubes A and B contained the virus of foot-and-mouth disease. It was therefore demonstrated that tube A contained only the virus of foot-and-mouth disease, tube B a mixture of the two viruses and tube C only the virus of

CHART 1.

EXAMINATION OF UNKNOWN SYSTEMS BY FILTRATION AND INOCULATION OF EGGS



vesicular stomatitis. A stock broth filtrate (membrane A.P.D. 0·8μ) was made of the virus collected from the guinea-pigs inoculated intradermally into the pads, with the suspension of the chorio-allantoic membranes of the eggs injected with a sample from tube B. A secondary filtrate, membrane A.P.D. 99 mμ., was made from this stock filtrate and proved to be inactive when inoculated into 2 guinea-pigs. The stock filtrate itself, however, when inoculated into eggs, produced lesions in eggs from which virus could be recovered by inoculation of guinea-pigs (Chart 1). These results showed that B, the mixture of the two viruses, had been resolved into its components.

The result of this experiment was remarkably clear-cut, and the time necessary to determine which virus or viruses were present in the " unknowns "

was 4 days. It must be pointed out, however, that the mixture contained equal amounts of each virus in high concentration, and that an unknown mixture might conceivably have one of its components in a lesser concentration than the other. Before proceeding with the examination of mixtures of this nature, an experiment was made to determine whether the two viruses could be passaged together in guinea-pigs.

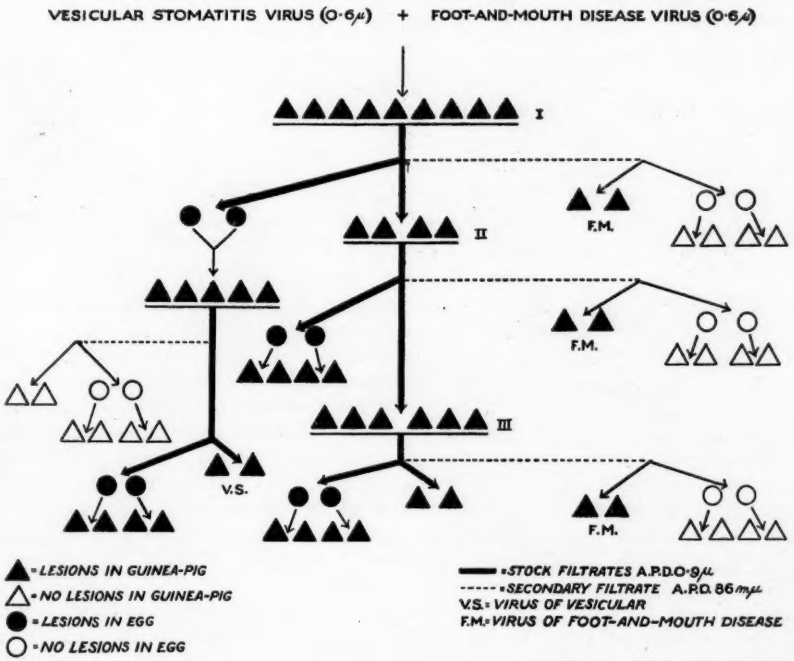
Experiment 2 (see also Chart 2) : Passage of the Two Viruses Together in Guinea-pigs.

Stock broth filtrates (membranes A.P.D. 0·6μ) of each virus were prepared, and a mixture was made so that the concentration of the two components was about the same. Quanta of this mixture were inoculated intradermally into the pads of 9 guinea-pigs. The vesicle lymph was collected at the 48th hour from these animals, diluted 1 : 10 in broth and a stock filtrate (membrane A.P.D. 0·9μ) made. A quantum of this stock filtrate was inoculated into the pads of each of 4 guinea-pigs. At the 48th hour the vesicle lymph was collected from these animals and treated in the same way as in the first passage. A third passage was made under the same conditions (Chart 2). At each successive passage a sample of the stock filtrates was injected into 2 eggs ; lesions developed in these, and virus could be recovered from them by inoculation of guinea-pigs. This showed that the virus of vesicular stomatitis was present, even after three passages in guinea-pigs. In addition, at each stage of the series of passages a secondary filtrate (membrane A.P.D. 86mμ.) was prepared from the stock filtrate. The secondary filtrates were inoculated into both eggs and guinea-pigs. The latter developed generalized lesions, while no lesions developed in the eggs or in the guinea-pigs subsequently inoculated with suspensions of the chorio-allantoic membranes taken from them. That all three secondary filtrates were infective showed that the virus of foot-and-mouth disease was present even in the third passage through guinea-pigs. At the first stage of the series of guinea-pig passages (Chart 2), the vesicle lymph was collected from the guinea-pigs which had been infected with the suspension of the chorio-allantoic membranes from the 2 eggs (these eggs had been inoculated with the first stock filtrate). A stock broth filtrate was prepared from this and then also a secondary filtrate. The stock filtrate and the secondary filtrate were injected into eggs and guinea-pigs. Lesions appeared in the eggs and guinea-pigs inoculated with the stock filtrate, but not in those inoculated with the secondary filtrate. Virus was recovery from the egg lesions by inoculation of guinea-pigs. The results of this experiment showed that under certain conditions both viruses can be passaged together in guinea-pigs. Confirmation was also brought to a fact which was demonstrated in Experiment 1, that it is possible to resolve a mixture of the two viruses by the method indicated.

Although both viruses survived three passages through guinea-pigs in this particular case, and therefore it can be assumed that an animal even under natural conditions might be infected with both viruses at the same time, successful passaging of the two viruses together was not attained in every experiment. When, for example, the vesicle lymph was collected from the

guinea-pig lesions at the 24th hour instead of the 48th, it was found that after several passages only the virus of foot-and-mouth disease could be recovered. This result might be partly explained by the fact that the incubation period in the guinea-pig infected with the virus of vesicular stomatitis is generally about 24 hours longer than in the case of foot-and-mouth disease. In passaging every 24 hours instead of every 48 hours the latter virus would be favoured, with the result that it would gradually become dominant and eliminate all traces of the other.

CHART 2.—*Separation of the Viruses of Vesicular Stomatitis and Foot-and-Mouth Disease after being Passaged Together in Guinea-Pigs.*



In yet another experiment the vesicle lymph diluted in broth and pads from infected guinea-pigs were stored for more than a few days in between certain of the passages and the virus of foot-and-mouth disease only was recovered. It is conceivable also that under some conditions more favourable to the virus of vesicular stomatitis, the foot-and-mouth disease virus might disappear from a mixture.

Variable results are to be expected in experiments of this nature, depending on the conditions. The essential fact which has emerged from these observations is that animals can be infected with both viruses simultaneously.

Experiments 3 *and* 4 *: Detection by Filtration of a Small Amount of the Virus of Foot-and-Mouth Disease in a Mixture of the Two Viruses.*

Experiment 3.—The strain of virus of vesicular stomatitis employed had been passaged only in guinea-pigs, and had not at any time been through eggs. Stock broth filtrates of both viruses were prepared. A series of tenfold dilutions in broth were made of the foot-and-mouth disease filtrate, and to each of these dilutions was added an equal amount of stock filtrate of the vesicular stomatitis virus (Table IV). Secondary filtrates (10 c.c. in amount)

TABLE IV.—*Detection by Filtration of a Small Amount of the Virus of Foot-and-mouth Disease in a Mixture of the Virus of Vesicular Stomatitis and the Virus of Foot-and-mouth Disease.*

Mixtures of stock filtrates.	Membrane employed for secondary filtrates.	Test on filtrates. Guinea-pigs.	Eggs.	Passage of eggs → guinea-pigs.
Undiluted V.S. N.T.E. + 1 : 10 F.M.	A.P.D. 0·13μ	++	00	00
Ditto + 1 : 100 F.M.		++	00	00
„ + 1 : 1000 F.M.		++	00	00
„ + 1 : 10,000 F.M.		++	00	00

Titre of stock vesicular stomatitis filtrate in eggs = 1 : 100,000.

Titre of stock foot-and-mouth disease virus in guinea-pigs = 1 : 10,000 + 0. / 1 : 100,000 + 0.

V.S. N.T.E. = Guinea-pig (only) passaged virus.

through membranes of A.P.D. 0·13μ were made from each of the mixtures, and quanta were inoculated into guinea-pigs and into eggs. All the guinea-pigs developed lesions, even those inoculated with a mixture of undiluted vesicular stomatitis virus and a 1 : 10,000 dilution of foot-and-mouth disease virus. (The end-point of the titration in guinea-pigs of the foot-and-mouth disease virus lay somewhere between 1 : 10,000 and 1 : 100,000.) None of the eggs inoculated respectively with the four secondary filtrates developed lesions, and no virus could be recovered from them by subinoculation into guinea-pigs. This result also confirms evidence obtained in experiments with guinea-pig (only) passaged vesicular stomatitis virus, described in Section I, *i. e.* such virus did not pass through membranes of A.P.D. 0·13μ (*vide* V.S. N.T.E., Tables I and II).

Experiment 4 was made in a similar way to Experiment 3, but membranes of a lesser porosity (A.P.D. 50 mμ.) were employed for the secondary filtration. The secondary filtrates were collected in two successive amounts of 5 c.c., and these were tested separately for virus by inoculation of eggs and guinea-pigs. Foot-and-mouth disease virus was detected even in the mixture of equal parts of a 1 : 10,000 dilution of that virus and undiluted vesicular stomatitis virus (see Table V). It appears that the presence of the vesicular stomatitis virus has but very little effect on the filtration of the virus of foot-and-mouth disease through membranes even of as low a porosity as A.P.D. 50 mμ. (*cf.* the above results with parallel filtrations of different dilutions of foot-and-mouth disease virus alone through membranes of a similar porosity see [Table V]), and it is therefore possible to detect the latter virus in a mixture by filtration even when present in an extremely low concentration.

TABLE V.—*Detection by Filtration of a Small Amount of the Virus of Foot-and-mouth Disease in a Mixture of the Virus of Vesicular Stomatitis and the Virus of Foot-and-mouth Disease.*

Mixtures of stock filtrates.	Membranes employed for secondary filtrates.	Test on filtrates. First sample, 5 c.c. Guinea-pigs.	Eggs.	Passage of eggs →guinea-pigs.	Second sample, 5 c.c. Guinea-pigs.	Eggs.	Passage of eggs →guinea-pigs.
Undiluted V.S. + 1 : 10 F.M.	A.P.D. 50 mμ.	++	00	00	++	00	00
Undiluted V.S. + 1 : 100 F.M.		++	00	00	00	00	00
Undiluted V.S. + 1 : 1000 F.M.		+0	00	00	00	00	00
Undiluted V.S. + 1 : 10,000 F.M.		+0	00	00	00	00	00
Undiluted V.S. + 1 : 100,000 F.M.		00	00	00	00	00	00
Controls.							
F.M. 1 : 1000	A.P.D. 50 mμ.	++	—	—	++	—	—
F.M. 1 : 10,000		00	—	—	++	—	—
F.M. 1 : 100,000		00	—	—	00	—	—

Titre of stock vesicular stomatitis filtrate on eggs = 1 : 100,000.
Titre of stock foot-and-mouth disease filtrate on guinea-pigs = 1 : 10,000.

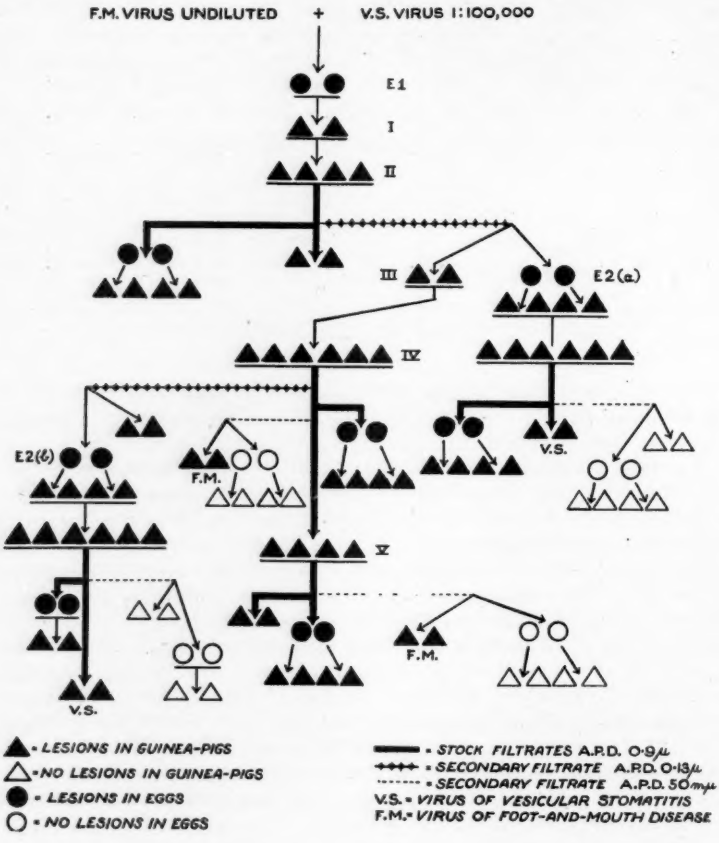
Experiment 5 : Detection of a Small Amount of Vesicular Stomatitis Virus in a Mixture of the Two Viruses and Final Resolution of the Mixture.

Stock broth filtrates (membranes A.P.D. 0·9μ) were prepared of the two viruses. The strain of vesicular stomatitis virus employed was that which had been used in Experiments 3 and 4, and up to the beginning of the present experiment had not at any time been passaged through eggs. A 1 : 100,000 dilution of the vesicular stomatitis stock filtrate was mixed in equal parts with the undiluted foot-and-mouth disease virus filtrate. A quantum of the mixture was inoculated into each of 2 eggs (Chart 3, E1), both of which developed lesions. Virus was recovered from a suspension in broth of the ground-up chorio-allantoic membranes of these 2 eggs and passaged twice in guinea-pigs (I and II, Chart 3).

It was necessary to determine whether any foot-and-mouth disease virus had survived the passage in the egg. A secondary filtrate (membrane A.P.D. 0·13μ) was therefore prepared, and this was inoculated into eggs (Chart 3 E2 (*a*)), in which lesions appeared, and into guinea-pigs, which also developed lesions. The positive result of the egg-inoculations meant either that the egg lesions were due to foot-and-mouth disease, which appeared unlikely, or that vesicular stomatitis virus (which had been passaged once through eggs (Chart 3, E1)) was present in the secondary filtrate (membrane A.P.D. 0·13μ). In view of the previous observations recorded in Section I of this paper, it seemed that the latter was the more probable. The chorio-allantoic membranes from the eggs (E2 (*a*)) were ground up and inoculated into guinea-pigs, which developed lesions. A second passage of the virus was made in guinea-pigs,

and then a stock filtrate (A.P.D. 0·9μ) and a secondary filtrate (A.P.D. 50mμ.) were made. Both filtrates were inoculated into eggs and guinea-pigs. The eggs and guinea-pigs inoculated with the primary stock filtrate developed

CHART 3.—*Detection of a Small Amount of Vesicular Stomatitis Virus in Presence of a High Concentration of Foot-and-Mouth Virus and Resolution of the Mixture.*



lesions; the eggs and guinea-pigs inoculated with the secondary filtrate did not, and no virus could be recovered from these eggs by subinoculation into guinea-pigs.

The observations up to this point showed that the vesicular stomatitis virus in an original concentration of 1 : 100,000 had been picked up by the eggs

(E1) inoculated with the mixture of the two viruses, and had also passed through the membrane of A.P.D. 0·13μ. This last result was unfortunate, as it did not help us in determining whether foot-and-mouth disease was present in the virus material recovered from eggs E1. The negative results of filtration through a membrane of A.P.D. 50 mμ. of the virus recovered from the eggs E2 (*a*) inoculated with the secondary filtrate (A.P.D. 0·13μ), however, showed definitely that no foot-and-mouth virus was present in this material. It had now to be determined definitely whether any foot-and-mouth disease virus had survived passage through the eggs E1 (Chart 3). The virus material collected from these eggs after four consecutive passages through guinea-pigs I, II, III, IV, was proved still to contain the virus of foot-and-mouth disease by secondary filtration through a membrane of A.P.D. 50mμ. The secondary filtrate was infective for guinea-pigs and non-infective for eggs. The presence of foot-and-mouth disease virus was also demonstrated in like manner in the fifth passage of this virus material in guinea-pigs (V, Chart 3).

A secondary filtrate (membrane A.P.D. 0·13μ) was prepared from the stock filtrate of the fourth guinea-pig passage (IV, Chart 3) of the virus material from the eggs E1. This secondary filtrate was infective for eggs E2 (*b*) and guinea-pigs. The virus, recovered from the eggs E2 (*b*) by guinea-pig passage, was proved to be free of foot-and-mouth disease by secondary filtration through a membrane of A.P.D. 50mμ. From a general consideration of the results recorded above and in Chart 3 the following interesting facts emerge: (1) The virus of vesicular stomatitis when present in very low concentration in a mixture with foot-and-mouth disease virus in very high concentration can still be detected by passage of the mixture in eggs. (2) Although foot-and-mouth disease virus when inoculated alone into eggs under standard conditions did not survive, when it was inoculated in high concentration simultaneously with the virus of vesicular stomatitis in low concentration some did survive one passage in eggs (see E1, Chart 3). A second passage through eggs (see E2 (*a*) and E2 (*b*), Chart 3) of the mixture of the two viruses sufficed, however, to eliminate the virus of foot-and-mouth disease, and vesicular stomatitis was recovered in the pure state. (3) Both viruses survived five consecutive passages together through guinea-pigs (V in Chart 3). (This confirms the observations made in Experiment 2.) (4) The strain of vesicular stomatitis virus (never passaged through eggs) employed in the present experiment was that which in four filtration tests in Experiment 3 did not pass through membranes of A.P.D. 0·13μ. Yet, after one passage in eggs, an active filtrate was obtained through a membrane of this porosity. (This result confirms those recorded in Section I.)

For choice in experiments of this type one would, of course, select a membrane just below the filtration end-point for the virus of vesicular stomatitis, say of A.P.D. about 100mμ. (This was done in Experiments 1 and 2, but it was not possible in Experiments 3, 4 and 5, as no membranes of this porosity happened to be available when these experiments were made, and the presence of foot-and-mouth disease virus had to be proved by employing membranes of A.P.D. 50mμ. That foot-and-mouth disease can be detected in very low concentration in a mixture of the two viruses by filtration through a membrane of this porosity was demonstrated in Experiment 4 [Table V].)

IV. STUDY OF THE RELATIVE STABILITY OF THE VIRUSES OF FOOT-AND-MOUTH DISEASE AND VESICULAR STOMATITIS AT DIFFERENT HYDROGEN-ION CONCENTRATIONS, AND INVESTIGATION OF THE SO-CALLED "REALKALINIZATION" TESTS FOR THE DIFFERENTIATION OF THESE TWO VIRUSES.

As a result of a series of interesting observations on the relative stability of the viruses of foot-and-mouth disease and vesicular stomatitis at different hydrogen ion concentrations, Pyl and Klenk (1933), Pyl (1934) and Reppin and Pyl (1934) have advanced a method of distinguishing between these two viruses by what they refer to as a "realkalinization" test.

It was decided first to observe the relative stability of the two viruses at different pH values. As far as possible methods similar to those employed by the authors quoted were adopted in the experiments about to be described.

Stability of the Virus of Foot-and-Mouth Disease at Different Hydrogen Ion Concentrations: Virus added 1 : 10 to Buffer Solutions at Required pH Value.

In the first series of experiments stock filtrates of the virus in physiological saline were diluted 1 : 10 in buffered solutions at various pH values ranging from about 1·5 to 10. Two series of tests were made under otherwise strictly comparable conditions at 0° C. and 22° C. In those tests made at 0° C., all the buffered solutions and the stock virus were placed at this temperature for about an hour before mixing, so that the various solutions were properly chilled. When samples were removed at intervals for testing they were inoculated intradermally into the pads of guinea-pigs as soon as possible after removal from the cold store, and in no instance did more than 15 minutes elapse between the two manipulations.

The results of several different experiments made at 0° C. are grouped together in Tables VI and VII, and those made at 22° C. in Table VIII.

The results of the long-period tests (see Table VI) did not agree with the observation, Pyl (1934), that the virus would survive for several days at about pH 3·0, and at this point we were inclined to doubt the validity of his conclusions.

The short-period tests at 0° C. and 22° C. (Tables VII and VIII) showed that some virus survived at a pH of about 3·0, but that the amount surviving was relatively less than that surviving at pH values from about 7·5 to 10, since only one guinea-pig out of two inoculated with the samples at about pH 3·0 developed lesions. No virus appeared to have survived at a pH of about 4·5 to 6·2.

The difference between the times of survival at 22° C. and 0° C. was only one of degree. By reference to Table VII it will be seen that under the conditions of the experiment the survival of some virus at about pH 3·0 might not be detected unless a series of tests at intervals was made. Indeed, in another experiment in which an arbitrary interval of 5 hours was chosen, a sample at about pH 3·0 was non-infective, and a sample at about pH 6·0 was infective.

TABLE VI.—*Stability of the Virus of Foot-and-mouth Disease at Various Hydrogen Ion Concentrations. Long-period Tests.* (*Virus Added* 1 : 10 *to Buffer Solutions at Required pH.*) *Temperature* 0° *C.*

pH of system.	Buffer system.	Time of test. 24 hours.	48 hours.	4 days.	5 days.	8 days.	13 days.
1·60	Citrate HCl *M*/10	00	00	—	—	—	—
1·60		00	00	—	—	—	—
2·93		00	00	—	—	—	—
2·95		+0	00	—	00	—	00
3·10		00	00	—	—	—	—
4·56		00	00	—	—	—	—
4·56		00	00	—	—	—	—
6·00		00	00	—	—	—	—
6·20	Phosphate *M*/15	00	00	—	—	—	—
6·23		++	00	—	—	—	—
6·24		00	00	00	00	00	—
7·50		++	++	++	—	++	—
7·60		++	++	—	—	—	—
7·71		++	++	—	—	—	—
7·71		++	++	—	++	—	++
10·00	Boric acid/KCl *M*/20	++	++	—	++	—	++
10·00		++	++	++	—	++	—

Remarks.

Titre of stock filtrates of virus in physiological saline 1 : 10,000–1 : 100,000.

The hydrogen ion concentrations of the various systems were estimated electrometrically after the addition of virus and again checked at the end of the experiment.

In the case of the system at pH 10, however, the hydrogen ion concentration was checked colorimetrically with thymol blue.

— = No test made.

++ = Both guinea-pigs inoculated developed lesions.

+0 = One of two guinea-pigs inoculated developed lesions.

00 = Neither of two guinea-pigs inoculated developed lesions.

TABLE VII.—*Stability of the Virus of Foot-and-mouth Disease at Various Hydrogen Ion Concentrations. Short period Tests.* (*Virus Added* 1 : 10 *to Buffer Solutions at Required pH.*) *Temperature* 0° *C.*

pH of system.	Buffer system.	Time of test. 2 hours.	3 hours.	4 hours.	5 hours.	6 hours.	10 hours.
1·60	Citrate HCl *M*/10	00	—	+0	—	00	00
1·60		00	—	00	—	00	—
2·93		+0	—	+0	—	+0	+0
2·95		+0	—	+0	—	00	00
4·56		00	—	00	—	00	00
4·56		00	—	00	—	00	00
6·00	Phosphate *M*/15	—	00	—	00	—	—
6·20		00	—	00	—	00	00
6·24		00	—	00	—	00	00
7·64		++	++	—	++	—	—
7·71		++	—	++	—	++	++
7·71		++	—	++	—	++	++
10·00	Boric acid/KCl *M*/20	++	—	++	—	++	++

Remarks.

Titre of stock filtrates of virus in physiological saline 1 : 10,000–1 : 100,000.

The hydrogen ion concentration of the various systems was estimated electrometrically after the addition of virus.

In the case of the system at pH 10, however, the hydrogen ion concentration was checked colorimetrically with thymol blue.

++ = Both guinea-pigs inoculated developed lesions.

+0 = One of two guinea-pigs inoculated developed lesions.

00 = neither of two guinea-pigs inoculated developed lesions.

— = No test made.

TABLE VIII.—*Stability of the Virus of Foot-and-mouth Disease at Various Hydrogen Ion Concentrations.* (*Virus Added* 1 : 10 *to Buffer Solution at Required pH.*) *Temperature* 22° C.

pH of system.	Buffer system.	Time of test. 1 hour.	2½ hours.	5 hours.	10 hours.	24 hours.	48 hours.
1·6	Citrate/HCl *M*/10	00	00	00	00	00	00
2·95		+0	+0	00	00	00	00
4·56		00	00	00	00	00	00
6·24	Phosphate *M*/15	00	00	00	00	00	00
7·71		++	++	++	++	++	++

+0 = One guinea-pig of two inoculated developed lesions.
00 = Neither of two guinea-pigs inoculated developed lesions.
++ = Both guinea-pigs of two inoculated developed lesions.

Stability of the Virus of Foot-and-Mouth Disease at Different Hydrogen Ion Concentrations: Virus in M/15 *Buffered Phosphate with pH Adjusted by Addition of* N/10 *HCl.*

In the second series of experiments the technique of adjusting the hydrogen ion concentration of the medium containing the virus was similar to that adopted by Pyl (1934) in his so-called "realkalinization" test to be described later. A stock filtrate of virus in *M*/15 buffered phosphate was prepared. The adjustment of the stock filtrate to the required pH value was effected by the addition of measured amounts of *N*/10 HCl. The *N*/10 HCl and virus filtrate were kept in the cold before the experiment so as to attain a temperature of 0° C., and all manipulations except the inoculation of guinea-pigs were made at 0° C. Samples were removed at intervals from the different systems for inoculation intradermally into the pads of two guinea-pigs. The results of the tests in different experiments are grouped together in Table IX. It will be seen that some virus survived at pH values from 2·48–3·64 even as long as 6, 7 and 10 days (6 experiments), while no virus was recovered from systems at pH values from 4·82–6·0. The results in this second series of experiments were much more regular, and moreover the virus survived for a very much longer period at about pH 3·0 than in the first series. Investigations of the cause of this difference indicated that it was to be attributed to the relatively detrimental influence of the citrate on the virus.

Experiments similar to those of the first series in which *M*/50 buffer solutions were employed instead of *M*/10, *M*/15 and *M*/20 solutions yielded results which, while they were more irregular, showed that the period of survival of the virus at about pH 3·0 was not affected by different concentrations of buffer salts.

These observations on the stability of the virus of foot-and-mouth disease at different hydrogen ion concentrations, in general, confirmed those made by Pyl and Klenk (1933), and Pyl (1934). The virus appears to remain infective over a considerable period at 0° C. at a pH of about 2·5–3·5, *i. e.* under conditions more acid than the generally accepted pH stability limit. The amount of virus which survives within this range pH 2·5–3·5 is, however, considerably less than within the zone pH 6·5–10. Generally the virus became rapidly inactivated within the zone pH 4·5–6·2.

TABLE IX.—*Stability of the Virus of Foot-and-mouth Disease at Various Hydrogen Ion Concentrations. Reaction of Virus in* M/15 *Buffered Phosphate Adjusted by Addition of* N/10 *HCl. Temperature* 0° *C.*

pH of system.	24 hours.	48 hours.	3 days.	4 days.	5 days.	6 days.	7 days.	10 days.
2·48	+0	++	00	+0	—	+0	—	—
2·67	++	++	++	+0	—	++	—	—
2·91	++	—	—	+0	—	—	—	—
2·90	++	++	++	+0	—	+0	—	—
3·05	++	++	++	—	—	—	—	—
3·07	++	++	++	—	—	—	++	+0
3·07	+0	+0	00	—	—	—	—	—
3·08	+0	++	++	—	—	—	+0	+0
3·08	++	+0	++	—	—	—	—	—
3·22	++	—	—	—	++	—	—	—
3·64	++	++	+0	+0	—	++	—	—
4·82	00	00	00	—	—	—	—	—
5·34	00	00	00	—	—	—	—	—
5·96	00	00	00	—	—	—	—	—
5·98	00	00	00	—	—	—	—	—
6·00	00	00	00	—	—	—	—	—

Remarks.

++ = Both guinea-pigs inoculated with sample reacted.

+0 = One guinea-pig out of two inoculated reacted.

00 = Neither of two guinea-pigs inoculated reacted.

Titre of stock virus filtrate in phosphate buffer solution employed = 1 : 10,000 to 1 : 100,000.

The hydrogen ion concentration of the various systems was estimated electrometrically at the end of each experiment.

TABLE X.—*Stability of Vesicular Stomatitis Virus at Various Hydrogen Ion Concentrations.* (*Virus Added* 1 : 10 *to Buffer Solutions at Required pH.*) *Temperature* 0° *C.*

Indiana strain. (Titre of stock filtrate = 1 : 1000.)

pH of system.	Buffer system.	Time of test. 2 hours.	4 hours.	6 hours.	10 hours.	24 hours.
1·78	Citrate/HCl *M*/10	00	00	00	00	00
1·78		—	—	—	—	00
2·96		00	00	00	00	00
3·02		—	—	—	—	00
4·52		—	—	—	—	00
4·52		00	00	00	+0	+0
6·20	Phosphate *M*/15	++	+0	++	++	++
6·20		—	—	—	—	00
7·59		—	—	—	—	+0
7·66		++	++	++	++	++
9·60	Boric acid/KCl *M*/10	++	++	++	++	++
9·60		—	—	—	—	++

New Jersey strain. (Titre of stock filtrate = 1 : 1000.)

pH of system.	Buffer system.	Time of test. 2 hours.	4 hours.	6 hours.	10 hours.	24 hours.
1·77	Citrate/HCl *M*/10	00	00	00	00	00
3·12		+0	00	00	00	00
4·51		++	++	++	++	++
6·01	Phosphate *M*/15	++	++	++	++	++
7·71		++	++	++	++	++
9·60	Boric acid K/Cl *M*/20	++	++	++	++	++

++ = Lesions in both guinea-pigs inoculated.
+0 = Lesions in one of two guinea-pigs inoculated.
00 = Lesions in neither of two guinea-pigs inoculated.
— = No test made.

Pyl and Klenk were of the opinion that the type of buffer systems employed did not affect the results, but our observations show that the time of survival at about pH 3·0 does vary when different methods of adjusting the hydrogen ion concentrations are employed. A satisfactory explanation of the existence of the two zones of relative stability of the virus of foot-and-mouth disease is not yet apparent, but the observations are strongly suggestive of a dissociation phenomenon determined by the prevailing hydrogen ion concentration and affected also by specific electrolyte ions, *e. g.* citrate. Pyl has suggested that the virus is associated with a colloidal carrier which may protect it within zones of hydrogen ion concentrations favourable to its survival, and from which it may become dissociated at certain pH values, viz. 6·0, when it is rapidly inactivated.

The obvious step towards acquiring a more complete picture of the phenomenon is to study the behaviour of purified preparations of the virus.

Stability of the Virus of Vesicular Stomatitis at Different Hydrogen Ion Concentrations : (1) *Virus added* 1 : 10 *to Buffer Solutions at Required pH Value, and* (2) *Virus in* M/15 *Buffered Phosphate with pH Adjusted by Addition of* N/10 *HCl.*

Investigations have been made with both the Indiana and the New Jersey strains of vesicular stomatitis. The methods employed were exactly the

TABLE XI.—*Stability of the Virus of Vesicular Stomatitis (Indiana Strain). (Adding Virus* 1 : 10 *to Buffer Solutions at Required pH.) Temperature* 22° *C.*

pH of system.	Buffer system.	Time of test. 1 hour.	2 hours.	4 hours.	24 hours.	Remarks.
1·77	Citrate/HCl *M*/10	00	00	00	00	Titre of stock filtrate in physiological saline = 1 : 1000.
3·03		00	00	00	00	
4·61		00	00	00	00	++ = Both guinea-pigs inoculated developed lesions.
6·19	Phosphate *M*/15	++	++	++	00	
7·64		++	++	++	++	00 = Neither of two guinea-pigs inoculated developed lesions.
9·7	Boric acid/KCl *M*/20	++	++	++	++	

TABLE XII.—*Stability of the Virus of Vesicular Stomatitis at Various Hydrogen Ion Concentrations. Reaction of Virus in* M/15 *Buffered Phosphate Adjusted by Addition of* N/10 *HCl. Temperature* 0°*C.*

Indiana strain. pH of system.	Time of test. 24 hours.	48 hours.	New Jersey strain. pH of system.	Time of test. 24 hours.	48 hours.
3·2	00	00	2·92	00	00
3·24	00	00	6·25	++	++
5·68	++	++			
5·89	++	++			
6·17	++	++	7·6	++	++
6·20	++	++			

same as in the experiments described above made with the virus of foot-and-mouth disease. Observations were made both at 0° C. and 22° C. The results are recorded in Tables X, XI and XII.

It is seen that neither strain of virus showed any tendency to survive at about pH 3·0, while both strains of virus survived at about pH 6·0 (*cf.* the virus of foot-and-mouth disease, which survives at about pH 3·0 and is inactive at about pH 6·0). Table X shows that the New Jersey strain of virus exhibited a slightly wider range of stability in acid solutions than did the Indiana strain. This difference between the two strains was also demonstrated in the "realkalinization" tests about to be described. Both strains of virus showed the same titre in guinea-pig tests, and in the actual experiments under discussion equivalent concentrations of the two viruses were always employed.

The zone of stability of the virus of vesicular stomatitis appears to be limited on the acid side at a pH of about 4·5. In contrast to the virus of foot-and-mouth disease no second zone of stability is found in a still more acid region.

The "Realkalinization" Tests as a Means of Distinguishing Between the Two Viruses.

Pyl (1934) and Reppin and Pyl (1934) have described what they call a "realkalinization" test, and have suggested that it can be used as a means of distinguishing the virus of vesicular stomatitis from that of foot-and-mouth disease. This test is based, in principle, on the observations that were made with regard to the difference in the relative stability of the two viruses at various hydrogen ion concentrations. The method of making the test originally described by Pyl is as follows: 0·1 c.c. of vesicle lymph is diluted in 10 c.c. of phosphate-buffer solution at about pH 7·7 and 1 c.c. of normal guinea-pig serum is added. To 5 c.c. of this virus suspension is added sufficient $N/10$ HCl (previously determined by titration) to adjust the pH of the virus system to about 3·0. "Realkalinization" to neutrality is effected by adding a corresponding amount of $N/10$ NaOH.

Pyl recorded observations which showed that, when the test was applied to the three standard strains of the virus of foot-and-mouth disease, the system was found to be inactive on "realkalinization" to neutrality. This was explained by the fact that during the process of acidification and subsequent alkalinization of the medium, the virus had twice passed through the zone pH 4·5 to 6·0 unfavourable to its survival. On the other hand, when the test was applied to either strain of the virus of vesicular stomatitis, the virus system was found to be active on realkalinization to neutrality. The time allowed to elapse before the acidified virus system was realkalinized did not appear to affect the results in Pyl's experiments.

In preliminary experiments on "realkalinization" made by the present writers with the virus of foot-and-mouth disease, it was found that the results were the same whether filtered or unfiltered virus was used (gradocol membrane

TABLE XIII.—*"Realkalinization" Tests with Foot-and-mouth Disease Virus and Vesicular Stomatitis Virus (Indiana and New Jersey Strains).*

Foot and mouth disease.						Vesicular stomatitis (Indiana strain).				Vesicular stomatitis (New Jersey strain).			
Dilution of lymph.	Time elapsing before "realkalinization".	System.	Tests. 10 min.	24 hrs.	5 days.	Dilution of lymph.	Time elapsing before "realkalinization".	System.	Test: 10 min. after "realkalinization".	Dilution of lymph.	Time elapsing before "realkalinization".	System.	Test: 10 min. after "realkalinization".
(A)		*Acidified*				(A)		*Acidified*		(A)		*Acidified*	
1 : 100	10 min.	pH 3·22	++	++	++	1 : 100	10 min.	pH 3·22	00	1 : 100	10 min.	pH 2·92	++
		"*Realkalinzied*"						"*Realkalinized*"				"*Realkalinised*"	
		pH 7·83	00	00	—			pH 7·78	++			pH 7·54	++
(B)		*Acidified*				(B)		*Acidified*		(B)		*Acidified*	
1 : 10	15 „	pH 2·86	++	—	—	1 : 100	15 „	pH 2·95	00	1 : 10	15 „	pH 2·94	++
		"*Realkalinized*"						"*Realkalinzed*"				"*Realkalinized*"	
		pH 7·57	00	—	—			pH 7·64	00			pH 7·5	++
(B)		*Acidified*				(B)		*Acidified*		(B)		*Acidified*	
1 : 100	15 „	pH 3·02	++	—	—	1 : 1000	15 „	pH 2·94	00	1 : 100	15 „	pH 3·07	+0
		"*Realkalinized*"						"*Realkalinized*"				"*Realkalinized*"	
		pH 7·57	00	—	—			pH 7·59	00			pH 7·4	++
										(B)		*Acidified*	
										1 : 1000	15 „	pH 3·02	00
												"*Realkalinized*"	
												pH 7·6	+0

Titre of stock virus A = 1 : 10,000.
„ „ „ B = 1 : 100,000.

Titre of stock virus A = 1 : 1000.
„ „ „ B = 1 : 1000.

Titre of stock virus A = 1 : 10,000±.
„ „ „ B = 1 : 1000.

filtrates). It was also found to be unnecessary to add normal guinea-pig serum to the virus system.

The results of " realkalinization " tests made with stock filtrates of the two viruses are recorded in Table XIII. All the experiments were done at 0° C. The time elapsing between the removal of samples for testing and the inoculation of guinea-pigs was never more than 10 minutes. In the case of the virus of foot-and-mouth disease, even if the concentration of the virus in the original system was greater than in Pyl's experiments, the " realkalinized " system was always found to be inactive. This confirms that author's observations. In the case of the virus of vesicular stomatitis, however, the position was somewhat different. The New Jersey strain gave more consistent results than the Indiana strain. In two experiments out of three with the latter strain the virus system was found to be inactive on " realkalinization ", although in one of them the dilution of virus was 1 : 100 as in Pyl's tests.

With the New Jersey strain, which of the two appears better able to survive for a short period in a system at about pH 3·0, an active liquid was obtained on realkalinization in all four tests, but there was evidence as in the case of the " Indiana " strain that the concentration of the virus influenced the final result, as did the time allowed to elapse before " realkalinization " was effected. It becomes apparent, therefore, that no accurate differentiation of the two viruses can be based on the " realkalinization " test. The final result of a test on the virus of vesicular stomatitis (especially the Indiana strain) may well be a non-infective system, as with the virus of foot-and-mouth disease, although the stage in the test at which the inactivation takes place is different. The virus of vesicular stomatitis may be inactivated at pH 3·0 before realkalinization is effected, whereas the foot-and-mouth disease is inactivated by passing twice through the zone pH 4·5 to 6·0. From our results, which generally confirm the observations of Pyl and Klenk (1933) and Pyl (1934), it may be concluded that there is a difference between the virus of foot-and-mouth disease and that of vesicular stomatitis as regards relative stability at different hydrogen ion concentrations.

It is the writers' considered opinion, however, that in view of the fact that variable conditions of experiments may influence the results and much accurate control of detail is therefore necessary, a reliable differentiation based on these observations is not possible.

SUMMARY AND CONCLUSIONS.

1. Vesicular stomatitis virus which has been passaged in eggs is found to filter somewhat more easily than virus which has been passaged only in guinea-pigs. This suggests that the virus undergoes some slight modification, either in size or surface properties, by passage in eggs. This modification, although definite and not easily reversible, is not of a sufficient magnitude to affect adversely the writers' original conclusion regarding the size of the virus (Galloway and Elford, 1933).

2. Employing a standard technique, which is described, the virus of foot-and-mouth disease was found not to survive even for 24 hours in a total of 58 developing hen's eggs injected (*cf.* the virus of vesicular stomatitis, which can be propagated in the chorio-allantoic membrane of developing hen's eggs [Burnet and Galloway, 1934]). The majority of these experiments were made with the G.F. strain of virus (Vallée and Carré O type), but a limited number of additional experiments with three other strains of virus, one of the Waldmann C type, one of the Vallée and Carré A type and the last of the Vallée and Carré O type yielded similar results.

3. It has been found possible to resolve mixtures of the viruses of foot-and-mouth disease and vesicular stomatitis irrespective of their relative concentrations by a method combining filtration with egg inoculation tests. The technique provides a straightforward and relatively economical method of rapidly and accurately distinguishing the two viruses and resolving a mixture of them.

4. It is possible, under experimental conditions at least, for an animal to be infected simultaneously with the virus of vesicular stomatitis and the virus of foot-and-mouth disease. Both viruses were passaged together successfully in guinea-pigs, and each virus was then recovered in the pure state by the method referred to above.

5. A study was made of the relative stabilities of the virus of foot-and-mouth disease and the virus of vesicular stomatitis at different hydrogen ion concentrations. The results obtained confirmed in general the observations of Pyl and his co-workers. The virus of foot-and-mouth disease remained infective over a considerable period at 0° C., at about pH 2·5–3·5, *i. e.* under conditions more acid than the generally accepted pH stability limit. The amount of virus which survived within this range, pH 2·5–3·5, was, however, considerably less than within the zone pH 6·5–10. Generally this virus rapidly becomes inactivated within the zone pH 4·5–6·2.

The zone of relative stability of the virus of vesicular stomatitis, on the other hand, appears to be limited on the acid side to about pH 4·5, and in contrast to the virus of foot-and-mouth disease no second zone of stability is found in a still more acid region.

In view of the fact that variable experimental conditions may lead to erratic results, it is essential that every detail be accurately controlled in any attempt to use these observations as a means of differentiation. Even so a differentiation based on them cannot be wholly reliable.

6. Reasons are given also for the conclusion drawn from experimental observations that no accurate differentiation of the two viruses can be based on Pyl's " realkalinization " test.

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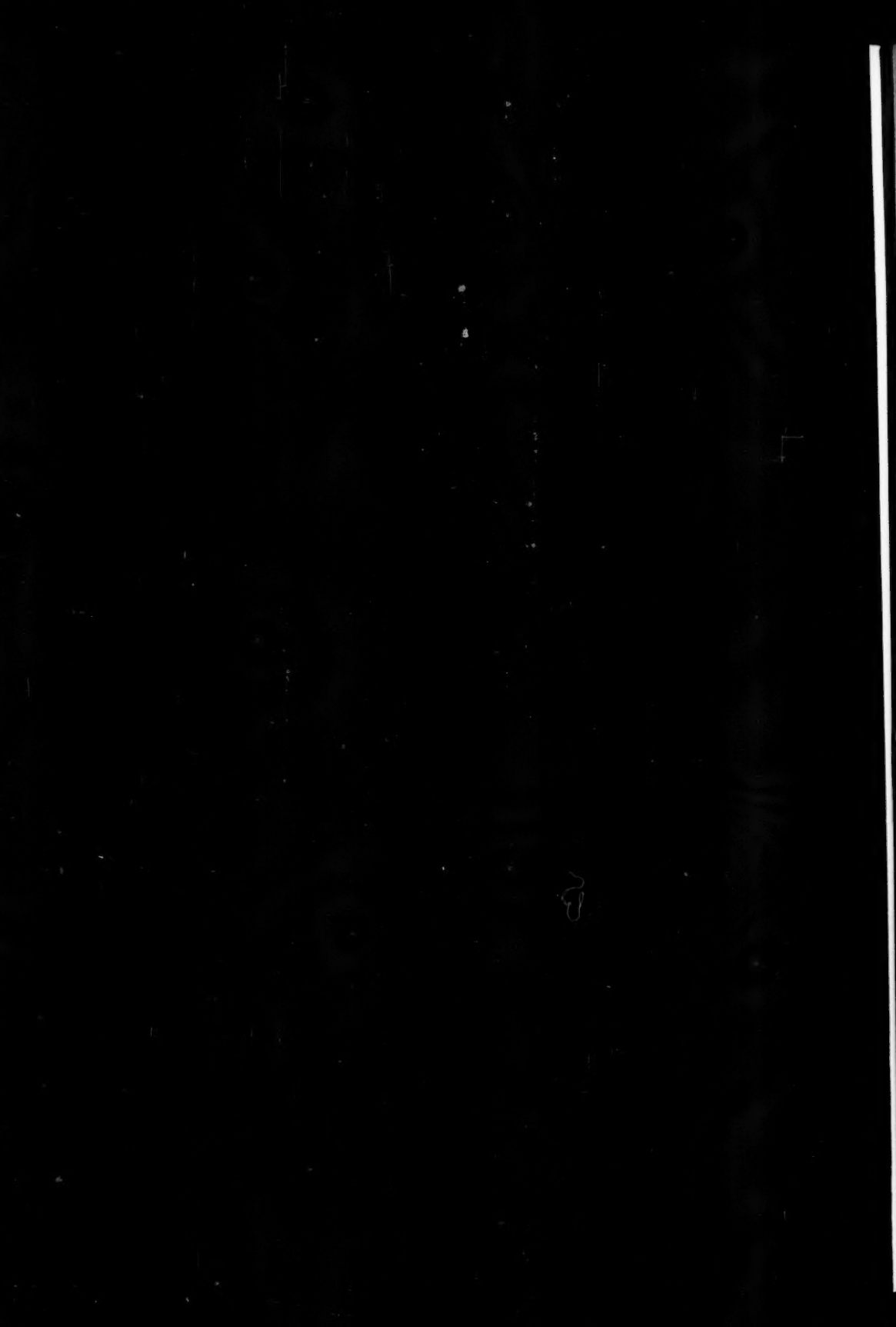

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